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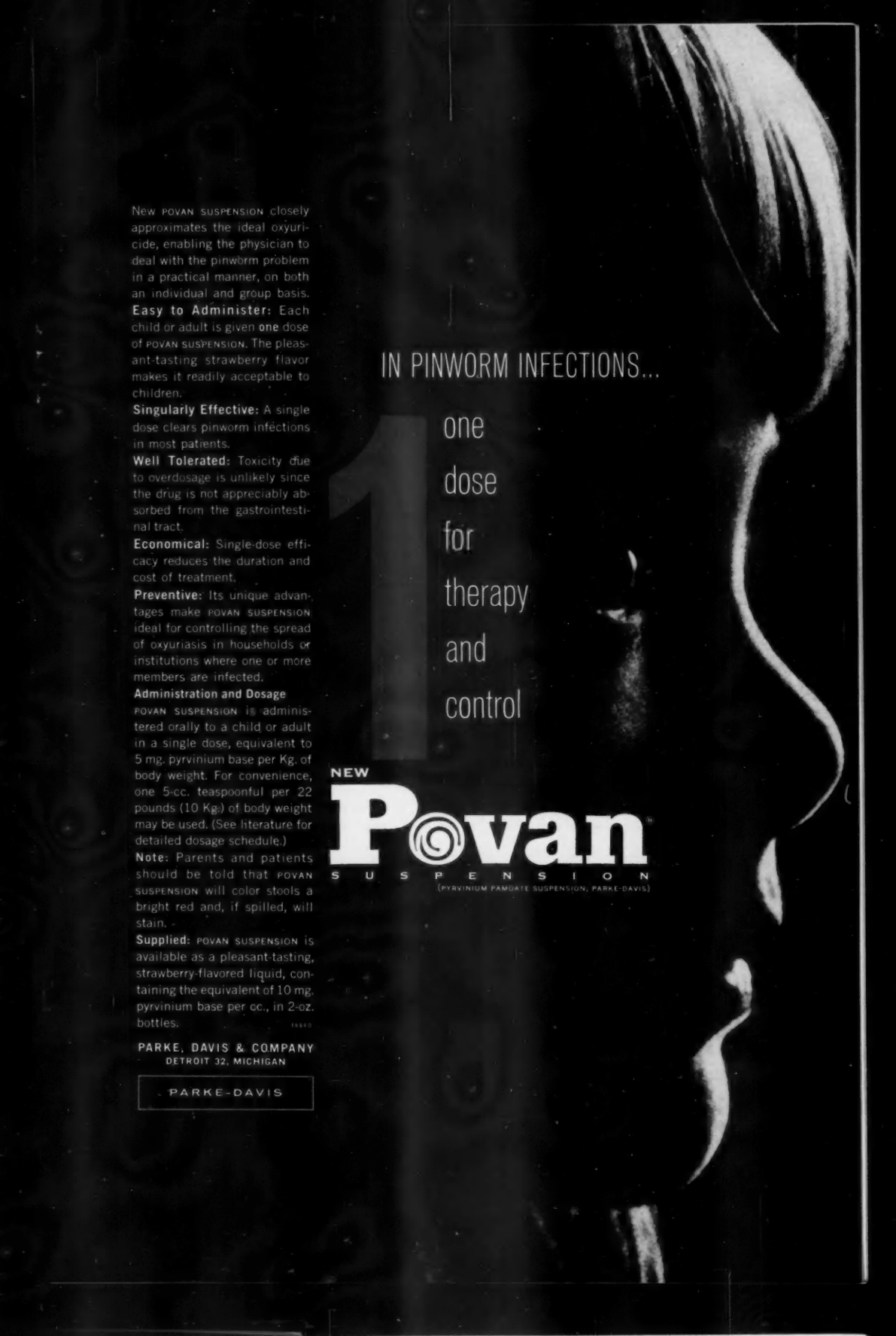


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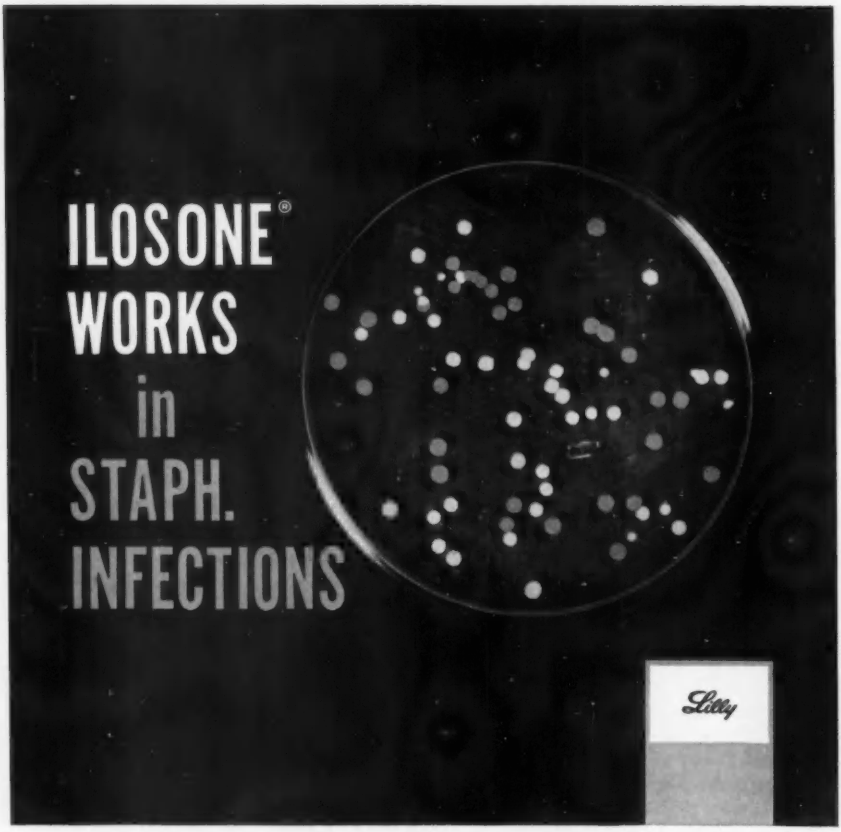


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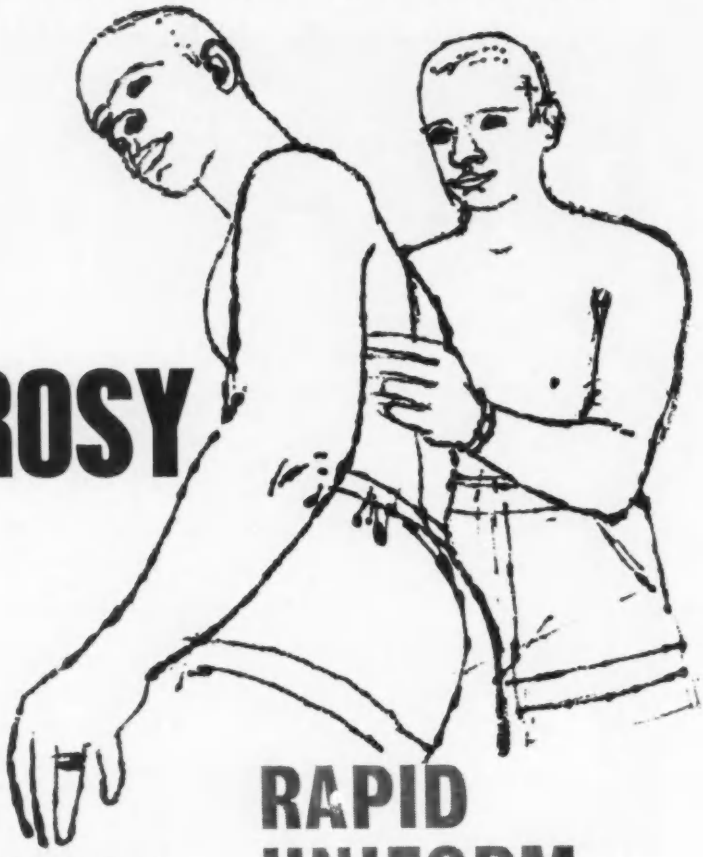

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"The first session was devoted to consideration of the complications and side-effects which occasionally result from one or other of the immunization processes, such as provocation poliomyelitis, encephalopathy occurring after the administration of pertussis vaccine, and generalized vaccinia and encephalitis resulting from smallpox vaccination. . . .

"In subsequent sessions, dealing with the different procedures which are now recommended and commonly practiced, opening and supporting papers were presented by those having special and in many cases unique experience.

"In the end . . . it was unanimously agreed to recommend the modified schedules (of immunization), a recommendation which, in view of the status of those who took part in the Symposium, must be regarded as giving authoritative guidance to those concerned.

"A final recommendation, well worthy of implementation, was that a representative body be set up to keep the problems of immunization under review and to make from time to time such recommendations as might be rendered necessary by advancing knowledge."—*From the Foreword by Brigadier Sir John Boyd, O.B.E., F.R.S.*

CONTENTS . . . First Session—The Risks of Immunization: Provocation poliomyelitis; Hazards of pertussis vaccination; Faults in the sterilization of syringes and needles. Second Session—Poliomyelitis Vaccination. Pertussis Vaccination. Third Session—Diphtheria Immunization. Tetanus Immunization. Combined Prophylactics. Personal Records of Inoculations. Fourth Session—Immunization Programs: Pros and Cons. Fifth Session—Discussion and Adoption of Recommendations. Appendix: Recommendations of the Symposium.

*Edited by* D. A. CANNON, *Medical Adviser, Wellcome Research Laboratories, Beckenham, Kent;* W. C. COCKBURN, *Director, Epidemiological Research Laboratory, Central Public Health Laboratory, Colindale, London;* D. G. EVANS, *Director, Department of Biological Standards, National Institute for Medical Research, London; and* H. J. PARISH, *Clinical Research Director, Wellcome Research Laboratories, Beckenham, Kent.*

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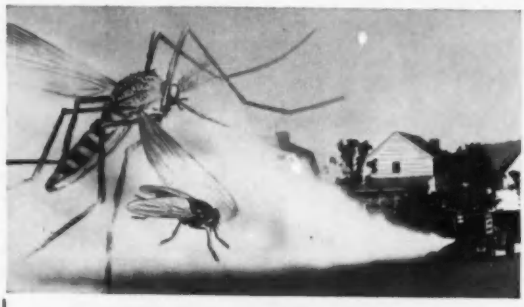

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# THE EPIDEMIOLOGY OF A DISAPPEARING DISEASE: MALARIA*

FRED L. SOPER†

The honor of the invitation to deliver the 24th Charles Franklin Craig Lecture is greatly enhanced for me by the presence of Dr. Lewis W. Hackett, my first chief, as our presiding officer. It is significant that the invitation to speak on Malaria Eradication comes so soon after the masterly 22nd Charles Franklin Craig Lecture on "Malaria Eradication—Growth of the Concept and its Application" by Dr. Louis L. Williams, Jr., just 2 years ago. This reflects the interest of the American Society of Tropical Medicine and Hygiene in what has come to be the greatest public health effort of all times and the recognition by your leaders that the development of the program for the world-wide eradication of malaria is so rapid that this Society must have frequent reports, if it is to discharge properly the responsibilities assumed in 1951 when the American Society of Tropical Medicine and the National Malaria Society were fused to become the American Society of Tropical Medicine and Hygiene. This Society must be the voice of the tropical public health workers of the United States in all matters related to malaria. The responsibility of this Society is indeed great since the economic resources for supplies and equipment and for the technical and administrative training of national staffs essential to eradication programs simply are not available in many of the most malarious areas of the world. The continued and increasing support of the United States Government through multilateral and bilateral agencies is indispensable. The responsibility of this Society as a source of informed professional opinion will increase as the world program continues to develop and as obvious successes in many areas lead to a false sense of optimism with a natural tendency to relax and reduce the all-out effort before the goal is reached.

The unusual procedure of inviting a Charles Franklin Craig Lecturer to speak on a specific subject followed, and may have been suggested by the remarks made a few months ago for the Washington Tropical Diseases Association after a trip to Asia with Dr. Robert Briggs Watson of the Rockefeller Foundation. Our trip to Asia was to observe at first hand eradication programs which, after early dramatic declines in malaria incidence, had failed to reach eradication on the anticipated schedule. We visited Taiwan, the Philippines, Ceylon and Mysore State, India, before attending the Third Asian Malaria Conference (March, 1959) in New Delhi where personal contact was established with many malaria workers from Southeast Asia and the Western Pacific. Later I visited the eradication program in Thailand alone. These countries were carefully selected as those which had solved the bulk of their malaria problem but were facing the problem of final extirpation of the infection.

The impressions from this all too rapid visit may be summarized as follows: 1. In each of the countries visited the introduction of residual insecticides was followed by a rapid decrease in malaria, almost to eradication, in large heavily populated areas during the first 2 years of overall coverage. 2. No serious technical difficulties were encountered in any of the areas visited: anopheles resistance to DDT was not a problem, nor was extra-domiciliary transmission of malaria proven. 3. The administrative difficulty of getting full coverage with residual insecticide of all human habitations in all of the malarious areas, and the epidemiological difficulty of identifying areas where transmission continues, both contributed to the failure to eradicate malaria rapidly.

There has been a failure to develop proper supervision and checking of spraying operations and to adapt the techniques suitable for heavily populated, easily accessible populations to fringe populations, to nomadic populations, and to scattered populations living in hilly and forested areas. Particularly has there been a failure in certain instances to recognize the necessity for spraying temporary shelters used by rural populations in connection with seasonal crops. There has been a tendency to be satisfied with the

* The essentials of this paper were given under the title, "Some 1959 Impressions of World-Wide Malaria Eradication," as the 24th Annual Charles Franklin Craig Lecture before the American Society of Tropical Medicine and Hygiene, Indianapolis, Indiana, October 28, 1959.

† Emeritus Director, Pan American Sanitary Bureau. Present address: 4101 Rosemary Street, Chevy Chase 15, Maryland.

general reduction of malaria, especially during the early years of the program, rather than to evaluate information from local communities to pinpoint places where malaria transmission continues. This blurring of the immediate objective of the eradication program, viz., the interruption of malaria transmission in each local community for the period required for spontaneous clearing of the infection in the human population, has led to costly delays, since partial eradication is always expensive.

In none of the countries visited had high blood indices persisted in the heavily populated, easily accessible areas, following the interruption of transmission. Nowhere was the charge of falsity made against the premise on which the eradication program is based. When properly used DDT had caused transmission to cease and malaria had disappeared rapidly.

The visits to the Asiatic eradication programs were entirely too short to do little more than gather general impressions from reading available reports, discussing details with program leaders and in some instances observing field operations. Surprisingly, each of the programs with minor variations seemed to fit the same pattern as outlined above. The analysis of the pattern indicates that the difficulties of malaria eradication are in general those of any eradication program and may be classed as, (1) administrative difficulties encountered in executing one hundred per cent of the necessary program, and (2) epidemiologic difficulties in identifying the places where the program has failed in its immediate objective and permitted transmission to continue. These difficulties seem to plague the health officer whenever he shifts from the concept of communicable disease control to eradication. The Public Health Administrator responsible for the control of communicable diseases, that is, reduction to the point where each disease is no longer a problem of public health importance in his community, tends to lose interest in the individual disease at the point where the eradicationist often encounters his greatest difficulty. This attitude is supported by international quarantine experience which shows that the threat of international movement of the infectious agents of communicable diseases is correlated with the epidemiological visibility of such diseases.‡ Also, the geographical area for which the Public Health officer is responsible is generally small and the threat of peripheral reinfection great, so it matters little that the seeds of infection remain in his area. For the eradicationist, continuing unrecognized transmission below the threshold of visibility constitutes the greatest threat to victory in the battle for complete elimination of the sources of infection.

The communicable disease control officer can take satisfaction from the absence of reported cases and of public clamor, but the eradicationist can be happy only when he has proven that transmission has ceased.

To emphasize the need for special study of the manner in which a communicable disease continues near or below the threshold of visibility and of the measures for its final suppression, I proposed some years ago the term, "The Epidemiology of a Disappearing Disease." I was gratified to receive an immediate cash award from Dr. L. L. Williams, Jr., who recognized the value of the term for this concept in malaria eradication. Dr. Louis I. Dublin has since used the term, "The Epidemiology of Retreating Tuberculosis," and Dr. Luis Vargas the term, "The Epidemiology of Evanescent Malaria."

The situation of malaria eradication in certain countries of Asia at the present time bears considerable resemblance to that of yellow fever eradication in Brazil in 1930. Since this is a Society of Tropical Medicine and Hygiene, and since today we are considering impressions and general principles rather than detailed statistical information as to the progress of malaria eradication in specific countries, we may well digress on the problems of yellow fever eradication in Brazil 30 years ago and the measures taken for their solution. This digression is entirely fitting since here in Indianapolis 59 years ago this month Walter Reed made the first presentation of the work of the Army Yellow Fever Board (Walter Reed, James A. Carroll, A. Agramonte, and Jesse W. Lazear) before the American Public Health Association.§

‡ Present day international sanitary regulations provide, in the case of smallpox, relapsing fever, cholera, typhus, and plague, that local infected areas can be declared free of any of these diseases when a period of only twice the incubation period of that disease has passed without the recognition of locally infected cases. This usage is based empirically on the observation that the chance of international movement of a given infection is relatively small when its incidence is so low that new cases do not come to the attention of the Public Health authorities.

§ Proceedings of the Twenty-eighth Annual Meeting of the American Public Health Association, Indianapolis, Indiana, Oct. 22–26, 1900.

By 1915 it had been observed that anti-mosquito measures in the important endemic centers of yellow fever in the Caribbean, Gulf and South American regions resulted routinely in the disappearance of yellow fever within a short period of time, not only in the endemic centers worked, but also in large tributary areas. General Gorgas logically concluded, and succeeded in convincing Wickliffe Rose of the International Health Commission of the Rockefeller Foundation, that anti-mosquito measures in the large cities of the remaining endemic areas of the Americas and of Africa would lead to the complete disappearance of yellow fever in the world. The initial efforts of the Rockefeller Foundation, collaborating with the health authorities of endemic countries of the Americas, were followed by the apparent disappearance of yellow fever from the west coast of South America, from Central America and Mexico, and from Columbia, and by 1925 the program for the eradication of yellow fever in Brazil, believed to be its last stronghold in the Western Hemisphere, was so far advanced that part of the Foundation's yellow fever staff was deployed to Africa to scout out the situation on that continent.

But yellow fever had not disappeared from Brazil, but had rather made a temporary tactical retreat in the face of the concerted onslaught in the large coastal cities of that country. Looking back from the vantage point of today this same pattern can be seen to have been repeated over and over again dating back to the first national eradication effort of the Brazilian Government. In 1916, General William C. Gorgas, as Chief of the Rockefeller Foundation's Yellow Fever Commission, had visited Brazil and been given evidence of continuing endemicity in the northeastern states. The entrance of the United States into World War I early in 1917 prevented the return of Gorgas to Brazil and interrupted the program of the Rockefeller Foundation. In 1918 the Brazilian Government, without waiting for Foundation participation, undertook the eradication of yellow fever as a national project. The work of the Federal Yellow Fever Commission, 1919 to 1921, throughout the northern states was an outstanding accomplishment. Yellow fever retreated on all sides and disappeared from the statistics of the area worked and in 1920 the Brazilian authorities saw no reason for accepting Foundation collaboration which was then available. In 1921, with yellow fever no longer a problem, the Brazilian Yellow Fever Commission became a rural health service with diversion of activity to hookworm disease, malaria, and other endemic conditions. In 1923, yellow fever reappeared and the Rockefeller Foundation undertook the organization of an eradication program in north Brazil. The rapid decline of yellow fever on all sides led once more to false optimism; a rude awakening came in 1926 when the movement of revolutionary and government troops through the interior led to widespread yellow fever outbreaks. Intensification of anti-mosquito measures over large areas followed and once more yellow fever receded precipitously from the statistics of the endemic area. In 1927–1928, a period of a year passed in which yellow fever was not recognized any place in the Americas. The reinfection of Rio de Janeiro, Brazil's beautiful capital, in 1928 from some unrecognized focus of infection, was followed by the widespread appearance of yellow fever along the coast from Rio to the Amazon Valley.

Anti-mosquito measures in Rio and the reorganization and intensification of such measures elsewhere led once more to the recession of yellow fever; but at long last it had become apparent that the premise on which the Rockefeller Foundation had embarked on the eradication of yellow fever was no longer tenable. It was obvious that the virus of yellow fever persisted unrecognized in spite of repeated anti-aegypti campaigns in all known endemic centers.

For almost three decades, *Aëdes aegypti* had been accepted as the one and only vector of yellow fever; apparent confirmation of the monopoly of transmission by aegypti had come repeatedly from the regularity of the disappearance of yellow fever from the health statistics of endemic and epidemic centers after the breeding index of this mosquito was brought below five per cent.

The first reaction following the appearance of yellow fever in Rio in 1928 was to attribute the persistence of yellow fever to administrative failures in the northern endemic foci. The maintenance of top efficiency in anti-aegypti work over long periods is a notoriously difficult administrative problem and gross imperfections were known to have occurred. Support for the suggestion that administrative failure might be responsible for failure to eradicate yellow fever came early in 1929 with the finding of yellow fever within 200 meters of the headquarters of the Yellow Fever Service in Recife, Brazil, when the reported aegypti-breeding index was only eight-

tenths of one per cent, far below the accepted safe threshold of five per cent. A check sample of 100 houses scattered at ten different points throughout the city gave an overall aegypti-index of twenty-six per cent; the percentage of houses with pupal foci, that is with foci actually producing adult aegypti-mosquitoes, was eight per cent. This result clearly indicated that the administration of the anti-mosquito work in Recife was inadequate and raised the question as to whether or not yellow fever had been able to maintain itself in the endemic centers themselves, where its vector had been under attack for a period of 5 or 6 years.

Beginning in 1930 the anti-aegypti services were completely reorganized and uniform operating procedures established throughout Brazil with adequate provision for checking, rechecking, and double checking the work done; and with cross checking by determining the results of anti-larval work on the density of adult aegypti, and the overall effect on the occurrence of yellow fever itself.

The most useful method of cross-checking the results of anti-aegypti work was found to be the search for adult mosquitoes in the houses of areas where low breeding indices were reported. The capture of adult mosquitoes, long a measure used by malariologists in a study of anopheline mosquitoes, had been largely abandoned by yellow fever workers because of the accessibility and ease of observation of larval breeding places in artificial water containers in and around human habitations. The capture of adult mosquitoes can be very misleading when the breeding index is high with an overlapping of distributional patterns from individual foci, but once the density of the species is really low the study of the distribution of adult mosquitoes, which can be caught in human habitations, tends to reveal often within a matter of a few meters where the guilty pupal focus lies.

The intensification and improvement of administrative procedures together with the introduction of the capture of adult mosquitoes for the discovery of hidden aegypti breeding resulted in 1933 in the complete disappearance or eradication of the *Aëdes aegypti* mosquito from some of the principal cities of north Brazil. This, the first observation of aegypti eradication during more than 30 years following the initial work of Gorgas in Havana in 1901, was of the utmost significance: (1) in the development of a rational long-term continental program against yellow fever; (2) as preparation for the eradication of *Anopheles gambiae* from Brazil and Egypt; and (3) in the acceptance of the concept of eradication in the prevention of communicable diseases.

A more fundamental cross check on the results of the work of the Yellow Fever Eradication Service than that provided by the search for adult aegypti was gotten through the systematic search for and diagnosis of unrecognized and unsuspected cases of yellow fever during silent inter-epidemic periods. Fortunately, the liver tissue of the victim of fatal yellow fever has microscopic changes by which the disease can be diagnosed; the search for yellow fever was based on the routine collection and examination of liver tissue throughout all possibly endemic areas, to which the name viscerotomy was given. Viscerotomy was to show that yellow fever had maintained itself in Brazil, not in one, but in two unrecognized forms, neither of which was related in any way to deficient administration of aegypti programs in the larger endemic centers. The first revelation of viscerotomy was of silent village and rural aegypti-transmitted endemic yellow fever in a large area of northeast Brazil in the hinterland just back from the coast where the Yellow Fever Service had been heavily engaged in combatting yellow fever in the port cities. This area was silent largely because the endemicity was so intense that the great majority of infections (and of fatal cases) were in children less than 5 years of age among whom mortality caused little comment. The discovery of this unsuspected endemic situation led to anti-aegypti measures in interior villages and even in strictly rural areas throughout several states of northeast Brazil. The last case of yellow fever diagnosed in this region occurred in August 1934; it is now 25 years since endemic, aegypti-transmitted yellow fever occurred in Brazil. Lowering the threshold of visibility of yellow fever in this area then led to the eradication of yellow fever just as lowering the threshold of visibility of aegypti-breeding led to the eradication of aegypti.

An even more surprising revelation of viscerotomy is the widespread distribution of yellow fever existing in the absence of the *Aëdes aegypti* mosquito throughout immense forested regions where yellow fever had never been recognized, or had not been reported for a decade or more. This

jungle yellow fever, which does not depend for its maintenance on man as the mammalian host, is associated with monkeys and other forest primates and has been shown since 1932 to be either enzootic or epizootic in large areas of the Americas ranging from northern Argentina to Mexico.

With the discovery of jungle yellow fever which constitutes a permanent source of virus from which the cities and towns of the Americas could be reinfected, it was realized that the eradication of yellow fever had been from the beginning an impossibility. It was recognized early that the discovery that the *Aëdes aegypti* mosquito could be eradicated, just at the time of the first finding of jungle yellow fever, was indeed fortuitous. The eradication of aegypti in Brazil's port cities was followed by its gradual but complete eradication from suburban villages and rural areas, and today there is a well advanced program for the complete eradication of the aegypti mosquito from the Western Hemisphere. Although the United States has not yet formally embarked on the eradication of *Aëdes aegypti*, the final result is inevitable since as each country frees itself of aegypti the pressure becomes greater on those which have yet to join in the continental program which is of permanent advantage to all.

No new administrative, technical nor administrative methods were involved in uncovering rural endemic and jungle yellow fever and in eradicating endemic yellow fever and the *Aëdes aegypti* mosquito. The improvement in administration involved the careful mapping of areas to be worked, the establishing of itineraries for working units, careful recording of all work at the time it was done, careful supervision and checking of work done, the standardization of operating procedures according to a written Manual of Operations, and the inculcation of the principle in all employees that they were responsible not only for doing their job, but also for getting the results expected from the doing of the job. The use of the capture of adult mosquitoes for the determination of continued breeding was not new, but had not been used for many years in yellow fever work. The identification through viscerotomy of places where yellow fever is present was developed 18 years after the diagnostic value of the liver lesion in yellow fever was established. Only the viscerotome was new; while it was an important factor in the success of viscerotomy its use was not indispensable to the method.

The malaria eradicationist today faces essentially the same problems that the yellow fever eradicationist did 30 years ago; how improve the administration to guarantee complete effective application of the chosen method of attack, and how identify, on a continuing basis and at a reasonable cost, the places where transmission occurs in spite of the attack? How determine the epidemiology of malaria when there has been such a great reduction in the incidence of the disease that cases are not being reported? And especially, how get these results economically? Given the essential identity of the malaria problem with that of yellow fever, it may not be amiss to outline briefly for the malariologist the way viscerotomy is organized in the Americas.

Viscerotomy was introduced when there was good reason to believe that there was no yellow fever in the large cities of Brazil; information was needed from rural areas, and eventually from the most isolated parts of the country. A consideration of the cost of maintaining full-time employees in hundreds, nay thousands of small communities in the interior, led from the beginning to the decision to appoint local representatives to collect liver tissue from indicated cases. The law provides that a burial permit, wherever a viscerotomist is located, must be approved by the viscerotomist before the body is interred. If it is ascertained that death has occurred in less than 11 days after onset of a febrile disease, the representative removes rapidly with a special instrument designed for the purpose (viscerotome) a small section of liver before approving burial. The viscerotomist receives a small fee for each specimen forwarded to the laboratory for diagnosis and a considerably larger one for the first one showing yellow fever lesions. He also receives a small fee for forwarding monthly the statistics from the local registrar which would otherwise not be available.

The results of viscerotomy have not been perfect; on the other hand the cost is not excessive and most of the information on the movement of yellow fever virus during the past three decades has come from this procedure. Viscerotomy does not pretend to the discovery of all cases of yellow fever since by its very nature, viscerotomy is limited to fatal cases. Rather viscerotomy identifies the place where yellow fever trans-

mission is present; of almost equal value is the negative evidence for the non-existence of yellow fever in certain large areas over a period of years. The information viscerotomy gives regarding jungle yellow fever often comes from the most isolated and difficult areas, where the cost of maintaining service employees would be inordinately high. The local representative has the advantage of being resident in the community and is there day after day, week after week, month after month. It is in just such areas as these that malaria eradicationists are now encountering their greatest difficulties.

Malaria eradication in various countries today seems to be following the pattern of endemic yellow fever eradication in Brazil from 1919 to 1934; in the face of an energetic frontal attack malaria disappears, recedes, evanesces, but remains to flare up and ridicule the eradicationist so soon as the attack is abandoned and eradication put to the critical test of the complete suppression of preventive measures. All too often the eradicationist is finding that the insecticidal work in some of his areas has been as poorly administered as had been the anti-aegypti work in Pernambuco in 1929 and that his epidemiological investigation has not shown him the silent malaria transmission that was continuing in his "jungle" areas. Without entering into details of anopheles resistance to insecticide and of plasmodial resistance to drugs, it can be said that the technical problems in malaria eradication would be much more easily solved were it possible to recognize every new infection at the time it occurs so that the failure to block transmission in each small delimited area, whether due to technical or administrative failure, could be denounced and corrected. The malariologist does not have any economical method of recognizing each new infection and may well have to settle, as did the Yellow Fever Service years ago, for the identification of the places where transmission is occurring.

Epidemiological evaluation as a check on the completeness of the interruption of transmission by early insecticidal spraying is hardly less significant than similar information in the terminal stages of the program when surveillance is taking over. Proper evaluation during the attack phase of eradication is essential to the decision to abandon spraying and begin surveillance; precipitate unjustified action can force a highly expensive reorganization whereas continued spraying when no longer required is equally wasteful. The malariologist must decide on the method of evaluation best adapted to his area: 1) routine systematic collection of blood slides from fever cases by local resident representatives; 2) routine collection of blood slides from fever cases by house visits of service employees at frequent intervals; and 3) mass or sampling surveys of all or of certain groups of the population without regard to history of fever.

### *Comments on Malaria Eradication in Certain Countries*

*Philippine Islands.* In the Philippines after some 4 years of widespread coverage with insecticidal sprays in areas with over 8 million people, field surveys were made (1957–1958) and interpreted to mean that no transmission had occurred during a 2 year period among some 5 million people living in previously malarious areas. Spraying was suppressed in 1958 in these areas. Surveillance was organized on the basis of house-to-house visits every 3 weeks with the examination of blood slides from all fever cases found and of persons reporting fever during the previous 3 weeks. The analysis of the findings at the end of 10 months led to the reorganization of spraying operations throughout much of the area where they had been suppressed. Most of the recrudescence of transmission found was attributed to the introduction of the infection in the "eradicated" areas from the sprayed areas where transmission had never been interrupted. Under Philippine conditions, it is apparent that it is not safe to discontinue spraying in one area until the areas from which it can be reinfected have also been cleared of infection.

*Ceylon.* Ceylon was one of the first countries in Asia to benefit from the introduction of DDT which has been widely used since the malaria epidemic of 1946–47. The government decided early in 1949 to attempt the complete eradication of malaria from the island and by 1954 considerable areas where transmission could not be found were removed from the spraying program. In many areas *Anopheles culicifacies* could no longer be found. During 1955 the search for fever cases in dispensaries was relied on to show where transmission was occurring; many of these dispensaries were themselves outside of the area of transmission. The search for fever cases in

dispensaries and the follow-up of these cases to identify the areas where transmission was occurring so that treatment and insecticide could be applied proved ineffective and in 1956–57 there was a considerable reinvasion of the "eradicated" area by malaria. In 1958 the spraying program was reorganized and today there is an intensified search for fever cases throughout the island and greater insistence on finding and spraying temporary shelters in the forest clearings. The success of the present program will depend on the seeking out and spraying of all human habitations rather than on finding every infected person and treating him and his contacts.

*Taiwan (Nationalist China).* Taiwan is another country in which DDT was introduced fairly early and from which excellent results have always been obtained. Here, although malaria disappeared in a large part of the heavily populated part of the island, the incidence of the disease did not continue to zero, as had been hoped for. An investigation of the situation in 1958 showed that transmission had ceased in the heavily populated agricultural parts of the island, but that in the hilly, mountainous areas with populations with scattered habitations of difficult access, transmission continued. Investigation showed that some of these areas had been poorly sprayed and some had been missed entirely in the general campaign on the island. Reorganization and intensification of the search for infective cases and of the spraying of isolated habitations have led to a month-by-month reduction in the number of cases found since September 1958 and it now appears that practically all of the transmission on Taiwan has been blocked and it can be anticipated that this country will become the first of the Asiatic countries to be able to declare the eradication of malaria.

*Thailand.* In Thailand the application of DDT has resulted in a rapid reduction of malaria and in the practical eradication of *Anopheles minimus* in much of the area covered by the spraying operations. Suppression of spraying has been possible in certain areas without the recurrence of malaria transmission. Impressive settlement of previously uninhabited areas has occurred. The program has, however, developed slowly with initial plans for protecting only the 13 or 14 million people living in the most malarious areas; it is, however, believed that another 9 million of the population live in areas exposed to risk. There is evidence that difficulties similar to those encountered in preventing transmission among peripheral fringe populations in other countries exist in Thailand and will become apparent with the expansion of the program to the forested areas. In summary, the program in Thailand is not sufficiently advanced to permit an appraisal of the problems to be encountered in the terminal stages of eradication.

*India.* The decision to undertake the eradication of malaria in India marked the inclusion of the largest single malarious population in the world in the eradication program. Although it was initially anticipated that anti-malaria measures would be applied in areas populated by some 225 million persons, it is now admitted that measures must be taken throughout areas inhabited by another 170 million people where malaria is relatively a minor problem. Although there are some reports of anopheline resistance to dieldrin, DDT seems to be giving excellent results. The program now being developed to complete the spraying of the homes of some 390 million people during 1960 represents a tremendous effort in financing and in administration. No serious technical difficulties are anticipated; the very size of the effort and the necessity of coordinating the programs of State services and of covering rural, nomadic and itinerant populations would seem to be the chief obstacles in India.

My remarks on malaria to this point have been devoted to the disappearance of malaria from parts of Asia. The term world-wide malaria eradication implies nothing less than the disappearance of all forms of human plasmodia. This disappearance must be not only from the Americas, from Asia and the Pacific, but also from the USSR, Communist China, Outer Mongolia, North Korea, North Vietnam, East Germany and from Africa. Some workers have tended to discount the possibility of malaria eradication in Asia since little was known of what was being done in those countries not members of the United Nations. In general, it would seem that the communist countries, with a governmental interest in the productivity of their populations and with relatively tight controls over their peoples, may be expected to eradicate malaria as fast as or even faster than

the free world, especially since most of these countries are not tropical.

In the USSR‖ a deliberate attempt to eradicate malaria dates from 1952 following an observed reduction in malaria incidence of almost 80% in 2 years. In the USSR, in addition to the spraying of homes with insecticide, all cases are given free treatment, are registered and supervised for a 2-year period. Malaria cases reported since 1950 by years are: 1950, 781,000; 1951, 351,000; 1952, 183,000; 1953, 116,000; 1954, 73,000; 1955, 36,000; 1956, 13,015; 1957, 5,095; 1958, 2,504.

To quote "... the malaria rate in the Soviet Union has been brought to such a low level that the country is on the threshold of complete eradication of this infection ... the presence of the high rate of malaria in other countries of the world, especially in the countries adjacent to ours, is of real concern to us. Introduction of malaria ... is at present gaining importance and increasing significance in the final period of local eradication of malaria.** ... The USSR shares this outlook as to the possibility and necessity for eradicating malaria throughout the world.

"The participation of the USSR in solving the malaria eradication problem in the world has not been limited solely to the framework of WHO. In 1955, The USSR Ministry of Public Health, at the request of the Government of the Democratic Republic of Viet-nam sent a group of Soviet malaria specialists to North Vietnam, where they worked for about three years... In 1956, a group of malaria specialists was sent to the Chinese People's Republic for the basic purpose of assisting in training local personnel. As a result of five months work ... a considerable number of Chinese specialists were trained."

The optimism and firm intent of the USSR malaria workers are apparent in the declaration that:

"The absence of new local cases for the absolute majority of regions in the country must be attained in the second half semester of 1959, and for the Azerbaidzhan SSR and Yukutsk of the ASSR not later than 1960."

Incomplete reports for 1958 showed 107 cases of malaria imported into the USSR, of which no less than 101 came from China. With regard to China I can do no better than quote from Dr. Williams' paper:

"Nothing specific can be said at this time of anti-malaria activities in Communist China but those familiar with her malarious areas perceive no technical difficulties standing in the way. Transportation difficulties are obvious in the various south-west provinces where terrain is rugged. However, roads are not entirely impossible and the local mosquito is notoriously susceptible to DDT."

In Africa, malaria eradication has been planned and is being attempted in a number of the more temperate countries, but there has been a certain reluctance to undertake eradication under present conditions in Africa south of the Sahara and north of Southern Rhodesia. The low economic level, the high rate of transmission, the shortage of trained personnel and the difficulty of communications in many parts of this region, when taken together, make the problem a formidable one. In spite of reported difficulties and local failures in the past, recent developments suggest

‖ Sergiev, P. G., Reshina, M. G., and Lysenko, A. Ya. Malaria as a world problem and progress in eradicating malaria in the USSR. *M. Parasitol. & Parasitic Dis.* (Ministry Health USSR), **28**: 268–280 (1959).

** This statement is highly significant. The lecturer has had the opportunity of observing the step by step development of the eradication of the yellow fever mosquito, *Aedes aegypti*, first from a number of the principal ports of Brazil, then the gradual peripheral spread of eradication to the suburbs, to the interior villages and even to the rural areas of northeast Brazil, to the Amazon Valley and eventually to the City of São Paulo and to the southern reaches of the country where urban yellow fever had never occurred but where the presence of the aegypti mosquito threatened eventual reinfestation of the eradicated areas. From Brazil's experience with reinfestations coming from her neighbors came the 1947 demand for continental eradication and Brazil's generous contributions in men and materials in carrying the program, through the auspices of the Pan American Sanitary Bureau, to other countries. Each of these in turn, as aegypti disappears from their territories, joins in the clamor for eradication in the territories of their neighbors.

As the global malaria eradication program advances, inevitably a residuum of malaria will be found in certain countries with low economic resources and inadequate administrative organizations, to whom the cost and difficulties of eradication may seem prohibitive. It is inevitable that more assistance will flow, if for no other reason as a matter of self-defense, from the more fortunate nations. As the number of nations without malaria increases, the demand to abolish malaria from all countries from which re-infection might come, will increase and there should be continually decreasing difficulty in getting staff and funds for the ultimate objective of final eradication from all countries.

there are no sound technical factors which will preclude success of any serious attempt to eradicate malaria when the time comes. A warning should be sounded against any attempt to eradicate malaria on a too limited basis in tropical Africa since reinfection from the periphery may be expected to be a more serious problem there than in other parts of the world. The creation of national independent governments throughout Africa may well create a demand for international participation in eradication programs for that continent much earlier than would have been the case had previous political conditions been maintained.

In the Americas, practically all of the malarious countries are engaged in the eradication effort. Serious difficulties with anopheline resistance to insecticides have been encountered in El Salvador and in Nicaragua and to lesser extent in other countries. Extra-domiciliary transmission seems to be a factor delaying eradication in certain areas of Venezuela, Costa Rica and possibly other countries. The great reaches of the Amazon Valley present a difficult problem in logistics; an attempt is being made to solve this problem with medicated table salt.

I have referred to my impression that the Malaria Eradication Program in many countries today is in a position similar to that of the Yellow Fever Eradication Program in South America 30 years ago. I believe it is not too much to anticipate that just as improvement in administration and in methods for rendering yellow fever and its vector visible resulted in a definite change in the situation for the better at that time, so may we anticipate that the next few years will see tremendous advances in the intensification and improvement of efforts for the eradication of malaria throughout the world. In India where the greatest reservoir of malaria infection has existed, one sees the development of a truly national effort and a national determination to eradicate malaria forever. One cannot fail to be impressed.

There are times when one can take heart from the perspective gained by attempting to look forward from a previous point in the calendar. May I quote for your consideration from a forward looking address of 15 years ago, the Presidential Address†† of 1945 for the National Malaria Society, one of the parent bodies of this Society:

"We are frequently reminded of the possibility of eradicating malaria in the United States, now that it is at a low ebb. I feel this is an untenable concept as we do not yet know in sufficient detail just where and under what conditions the disease occurs, or will occur in its last natural habitant. Possibly malaria will be eliminated but I much prefer to entertain the hope that we will build malaria out in our future developments and that we will attempt to 'reduce' rather than 'eliminate' it in its existing natural setting . . . it is unwise . . . to put malaria control operations into practice unless the disease is causing a measurable economic loss and unless the cost is in a measure commensurate with the economic ability of the people to pay."

But even as the President of the National Malaria Society was speaking, the die had been cast, and the budget item approved for the extended Malaria Control Program which was to become in 1947 the National Malaria Eradication Program. And indeed in 1950 and 1951 the National Malaria Society took steps for its own dissolution, as no longer necessary.

The action of the XIII Pan American Sanitary Conference in 1950 in recommending national programs for the eradication of malaria throughout the Americas was apparently ahead of its time and little stir was created until after the action of the XIV Pan American Sanitary Conference in 1954. The decision of Mexico to undertake a national malaria eradication program led to support of this program by UNICEF and the action of the Joint UNICEF/WHO Health Policy Committee approving malaria eradication for joint effort of the two organizations. This was followed almost immediately by the action of VIII World Health Assembly declaring for a world-wide malaria eradication program. The action and reaction of the 1954–59 period have been explosive in character in comparison with the slow speed at which international activities usually develop.

In 1957 the Pan American Health Organization received generous contributions to its Malaria Eradication Special Account from the governments of the Dominican Republic, the United States and Venezuela. In the same year the WHO received a sizeable contribution from the United States and the International Cooperation Ad-

†† Johnson, Henry A. Malaria in the Post-War Era. *J. Nat. Malaria Soc.*, **5**: 1–6 (1946).

ministration began to participate officially in malaria eradication with funds earmarked for the purpose by the United States Congress. The ICA is participating in malaria eradication in some two dozen countries, including India which has the world's largest population residing in malarious areas.

The governments of nations throughout the world have been most enthusiastic in girding themselves for the task of financing the internal costs of eradication; the difficulties have been related to trained professional staff and international funds to cover materials which must be imported.

The program for the eradication of malaria in the world has implications far beyond the economic, social, health, and cultural effects of this disease. Once the pattern has been set for international collaboration on a world-wide basis in the eradication of a single disease, it is obvious that the road is open for similar action on other human, animal, and plant diseases, and insect and plant pests. On October 22, 1959, the Secretary of Agriculture announced the eradication of vesicular exanthema of swine in the United States. Vesicular exanthema had been present for some 20 years in California in a known but local focus of infection, which suddenly and unexpectedly had an opportunity to spread, apparently on dining cars on inter-state railway trains some 7 or 8 years ago. At its peak, forty-three states were known to be infected and embargoes were placed on the importation of United States pork products by ten countries: Canada, the United Kingdom, Columbia, Venezuela, Austria, Belgium, Sweden, Barbados, Jamaica, and British Guiana. California is now free of the infection at the price of having infected the other forty-two states and at the price of a 7 year campaign waged by the Department of Agriculture in collaboration with the state governments. In making this announcement Secretary Benson did not announce a program of co-operation with the ten clean countries which embargoed pork from the United States to help rid the rest of the world of vesicular exanthema, but might well have done so had malaria eradication been completed.

Recent success in the dramatic eradication of the "screw worm" from its eastern range of distribution in Florida and Georgia leads one to the question: "If Florida has no 'screw worm' can Texas be far behind?" Eradication of the screw worm in Florida was possible because of the isolation of the eastern focus from other countries by the Gulf of Mexico and the Atlantic Ocean. Texas is not in a similarly favorable position and eradication there would put the United States in a defensive position, vís-à-vís the infested areas of Mexico. As Mexico proceeds in its program for the eradication of the *Aëdes aegypti* mosquito, the urban vector of yellow fever from its territory, it faces a similar problem along the border with the United States and in its contacts with gulf ports. Well may the representatives of agriculture and of health of the two countries hold a combined meeting and arrange broad collaboration in the solution of both problems.

It may be well to close this Charles Franklin Craig lecture by quoting the words of the Secretary of Health, Education, and Welfare, when on October 20, 1959 he presented at the annual meeting of the American Public Health Association, contributions of two and of three million dollars respectively to the Malaria Eradication Special Accounts of the Pan American and World Health Organizations:

"The cause to which this money will be applied is a triumphant one. Never until very recent times has man dared to talk of 'eradication.' In all war against disease we have moved from helplessness to treatment and thence to prevention and control. As we move toward the eradication of a disease from the face of the earth we stand on the threshold of total victory for man over one of his oldest and deadliest enemies."

But total victory over malaria can come only as there is total coverage of infected populations and as malaria is not permitted to become a Disappearing Disease before it has been eradicated.

# TRIAL OF AN INDIRECT HEMAGGLUTINATION TEST FOR THE DIAGNOSIS OF INFECTIONS WITH *CLONORCHIS SINENSIS**

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Detection of infections with *Clonorchis sinensis* has been accomplished through sero-diagnostic procedures using various extracts of the parasite. Thus far, however, only complement fixation (CF) and intradermal tests (ID) have been employed for this purpose. In 1921 Ryuji showed that an alcohol extract of rabbit livers infected with *C. sinensis* could be used effectively in a CF test for detecting experimental infections in animals.[1] The titers obtained from this test were relatively low even for sera from heavily infected rabbits; sera from human beings with light infections were negative.

Saline extracts of *C. sinensis* were found by Ritchie and associates to cross react in ID tests of patients infected with *Paragonimus westermanni* and *Schistosoma japonicum*.[2] Chung, working with saline extracts of *Clonorchis* and *Paragonimus*, found cross reactivity both in CF and in ID reactions.[3] The same author later extended his observations to include cross reactivity with *Fasciola hepatica*.[4] A more sensitive and specific extract was prepared by Wykoff[5] for use in the CF test, using Chaffee's method of removing lipids.[6] More recently, work by Sadun and associates has shown that two protein fractions of *C. sinensis* are sensitive and specific antigens when used in CF and ID tests.[7]

Antigen-antibody systems in infections with such helminths as *Schistosoma*,[8] *Trichinella*,[9] *Echinococcus*,[10-12] and ascarids (*Ascaris* and *Toxocara*),[13-17] as well as several bacterial infections, have been successfully demonstrated by using the tanned-cell indirect hemagglutination (IHA) test. It was thought desirable to determine whether this test would prove equally sensitive in demonstrating specific serum components in rabbits and human beings infected with *C. sinensis*.

## MATERIALS AND METHODS

Kagan's modification[7] of the Boyden technic of IHA[18] was used. Sheep red blood cells were washed, tanned and subsequently coated with the worm extract. The tanned sensitized red blood cells were then mixed with the serum to be tested and the mixture was allowed to stand at room temperature for approximately 3 hours, after which time the tests were scored. The tubes were shaken and allowed to remain overnight. Final scoring, based on cell sedimentation patterns, was carried out according to the method of Salk.[19]

Two extracts were used in the test. The first of these, used previously by one of us (DEW) in a CF test,[5] was a triethanolamine buffered saline (TBS—pH 7.3 to 7.4) extract of lyophilized adult worms previously ground twice in ether at −18°C according to the method of Chaffee and associates.[6] The second was a phosphate buffered saline (PBS—pH 7.2) extract prepared by grinding 100 mg of lyophilized adult worms in 10 ml of PBS in a chilled tissue grinder. The ground material was transferred to another container and the tissue grinder was rinsed with an additional 5 ml of PBS which was added to the original volume. After extracting in the cold (5°C) with constant stirring for 4 hours, the material was centrifuged at 850 × G for 30 minutes. The supernatant fluid was removed and lyophilized in ampules which were sealed off under vacuum. During preparation of both extracts the temperature was maintained at or below 5°C to minimize denaturation.

The optimal dry weight concentration of extract for routine use was determined by titrating each against known positive sera. The optimal concentration was considered to be one which resulted in measurable, reproducible titers of positive serum while normal serum and diluent controls were negative.

The sera used for these studies were from 4 sources: (1) rabbits bled weekly over a period

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TABLE 1

*Results obtained from testing two* Clonorchis *extracts against homologous and heterologous sera*

Reciprocals of maximum titers obtained from individual serum samples tested with extracts "A" and "B"

| Sera from rabbits and humans infected with *Clonorchis* | | Sera from humans, a cat (c) and rabbits (r) infected with | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| (Rabbits) | | (Humans) | | *Paragonimus* | | *S. mansoni* | | *S. japonicum* | | *S. haematobium* | |
| A* | B† | A | B | A | B | A | B | A | B | A | B |
| 5120 | 1280 | 2560 | 80 | <10 | 40 | <10 | 20 | <10 | —‡ | <10 | 40 |
| 5120 | 1280 | 640 | 80 | <10 | 20 | <10 | 10 | <10 | — | <10 | 40 |
| 5120 | 640 | 320 | 80 | <10 | <10 | <10 | 20 | <10 | — | <10 | 10 |
| 5120 | 1280 | | | <10 c | 40 c | <10 | <10 | <10 r | 20 r | <10 | — |
| 160 | 80 | | | | | <10 | <10 | <10 r | 20 r | <10 | — |
| 320 | 320 | | | | | | | | | | |
| 320 | 320 | | | | | | | | | | |

* Triethanolamine buffered saline extract of ether treated, lyophilized adult whole worms used at a dry weight concentration of 9 μg/ml.

† Phosphate buffered saline extract of lyophilized adult whole worms used at a concentration of 36 μg/ml.

‡ Not tested.

of 55 weeks following experimental inoculation with 300 metacercariae of *C. sinensis*, these sera having been used in a previous study and stored at −50°C for approximately 1 year;[5] (2) human beings infected with *C. sinensis*; (3) human beings infected with *Ascaris lumbricoides*, *Trichiuris trichiura*, *Necator americanus*, *Strongyloides stercoralis*, *Paragonimus westermanni*,‡ *Schistosoma mansoni*,§ *S. japonicum*,‡ or *S. haematobium*;‖ and (4) animals experimentally or naturally infected with *Toxocara canis*, *A. lumbricoides*, *Heterobilharzia americana*, *Paragonimus kellicotti*‡ or *S. japonicum*.‡

## RESULTS

The optimal dry weight concentration of the PBS extract was found to be 36 μg/ml when tested against homologous rabbit sera. Cross reactivity was found when this extract was tested with sera from human beings infected with *P. westermanni*, *S. mansoni* and *S. haematobium* as well as with sera from animals infected with *P. kellicotti* and *S. japonicum* (Table 1). No reaction was found with sera from human beings infected with *A. lumbricoides*, *T. trichiura*, *N. americanus* or *S. stercoralis*. Similarly, no cross reactivity was found with sera from animals infected with *T. canis*, *A. lumbricoides* or *Heterobilharzia americana*.

The optimal dry weight concentration of the ether treated TBS extract was found to be 9 μg/ml. No cross reactivity was found when this extract was tested with any of the above heterologous sera (Table 1).

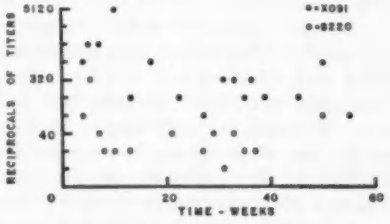


FIG. 1. Reciprocals of titers obtained from a tanned-cell indirect hemagglutination test using serum collected periodically during 55 weeks from 2 rabbits (X091, 8220) each of which had been experimentally infected with 300 metacercariae of *Clonorchis sinensis*. The sera were reacted against a buffered saline extract of ether treated adult worms that had been lyophilized from the living state. The sera and ether treated extract had been used previously in a study of the complement fixation test for clonorchiasis[6] and stored at −50°C for approximately 1 year.

‡ Sera supplied by Dr. E. H. Sadun.
§ Sera supplied by Dr. L. S. Ritchie.
‖ Sera sent from NAMRU III, Cairo, Egypt.

Changes in antibody titer in a 55 week experimental infection of 2 rabbits were determined by using the ether treated TBS extract (Figure 1). Pre-infection sera were all negative; antibody titers became detectable during the 4th week and reached a peak between 6 and 16 weeks of infection. Thereafter, the titers decreased and remained at a low level for the rest of the experimental period.

## DISCUSSION

As mentioned previously IHA has been shown to be a very sensitive test for the detection of various infections.[8-18] This is borne out by the results of this study. A comparison of the findings of the present study with those obtained by Wykoff[6] using the same extract (ether treated TBS extract) and the same homologous rabbit sera shows that in the CF test the optimal extract concentration was 225 μg/ml whereas in the IHA test it was 9 μg/ml. At these respective concentrations the maximum titer obtained from the CF test was 1:128 whereas in the IHA test the maximum titer was 1:5120. Thus in the IHA test less extract (4%) produced a titer 40 times greater. The present demonstration of greater sensitivity is in agreement with trials of similar tests for echinococcosis.[10-12]

Specificity of sero-immunological reactions in parasitic infections, when the parasites themselves are used as a source of antigen, has been shown to be enhanced by the purification of extracts.[5-9, 13-17] Additional evidence of enhanced specificity is provided by the present study in which it was found that an ether treated TBS extract was more sensitive and more specific than a simple PBS extract. It is of interest that none of the sera from animals or human beings with nematode infections reacted with either of the *Clonorchis* extracts tested despite the fact that high titers were obtained with these sera when tested with nematode extracts. However, the number of sera tested from animals or human beings infected with heterologous trematodes was small and no information was available as to when in the course of infection the blood was drawn. Therefore, final conclusions as to the cross reactivity of the extracts must await further controlled testing.

The relationship between the antigen-antibody systems demonstrated by IHA and by CF has not been determined. The antibody titers found by IHA in experimental infections in rabbits appear to rise early in the infection, reaching a peak between the 6th and 16th weeks and then decreasing. A similar rise and fall of antibody titers has been reported by Sadun and associates[7] and by Wykoff[6] using a CF test.

## SUMMARY

An indirect tanned-cell hemagglutination test was used for the detection of *Clonorchis sinensis* infections in man and rabbits. A comparison of the results from this test with those obtained earlier from a complement fixation test in which the same extract and the same sera were used showed the tanned-cell hemagglutination test to be relatively more sensitive. Specificity as well as sensitivity of the test was found to be increased by treating the dried worms with ether prior to saline extraction. With the ether treated material cross reactions were lacking in tests of a limited number of sera from animals and humans with *Schistosoma* and *Paragonimus* infections.

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# THE RELATION BETWEEN SCHISTOSOMIASIS AND THE URINARY *SALMONELLA* CARRIER STATE

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The incidence of *Salmonella* carriers in Egypt is very high. Miller[1] and Weir *et al.*[2] found among the population of an Egyptian village a carrier rate of 3.1%. In another village a recent survey by the Institute of Research for Tropical Medicine showed that 3.7% of the male inhabitants were chronic urinary carriers of either typhoid or one of the paratyphoid organisms.

Whereas in other parts of the world intestinal carriers are almost invariably more frequently found than urinary carriers, in Egypt the picture is reversed. In many surveys abroad the ratio of fecal to urinary carriers was 10 to 1.[1] Saphir *et al.*[3] reporting on 110 typhoid carriers in the United States found that only 2 of them were urinary carriers and both were at the same time fecal carriers. In Egypt, Miller[1] found that among 15 enterica carriers 10 were urinary, 4 were fecal and one passed the organism in both the urine and stools. The Weir group also found that among the chronic carriers the ratio of urinary to fecal was 7.1 to 1. There is a high incidence of urinary schistosomiasis in urinary *Salmonella* carriers. Miller and Floyd[4] found that all of 15 chronic urinary carriers had a history of schistosomiasis and were passing ova in the urine. Neva[5] reporting on 14 urinary carriers demonstrated *Schistosoma haematobium* ova in 8 of them, while an additional 3 had a definite history of schistosomiasis. Messih[6] reported on 3 urinary *Salmonella* carriers and mentioned that 2 had active schistosomiasis. The above results might be interpreted as showing a relationship between urinary schistosomiasis and the *Salmonella* carrier state, but in making such an interpretation caution should be exercised and due consideration should be given to the high incidence of urinary schistosomiasis in the general population.

This work is an attempt to determine the relationship between *S. haematobium* infection and the urinary *Salmonella* carrier state by studying the effect of specific treatment of the carrier state in patients with and without *S. haematobium* infection; and the effect of treatment for schistosomiasis on the response of carriers to specific treatment. To our knowledge this method of approach has not been previously utilized, though Messih[6] mentioned one patient who had schistosomiasis and continued to be a *Salmonella* carrier after treatment for schistosomiasis, and Abdalla[7] mentioned that 3 patients with both urinary schistosomiasis and *Salmonella* infection remained positive for the latter after successful treatment for the schistosomiasis. A preliminary report of the present study has been published previously,[8] and now the entire work is presented.

## MATERIALS AND METHODS

Three groups of *Salmonella* carriers were studied: Group I consisted of 12 chronic urinary carriers, negative for schistosomiasis, who were given chloramphenicol, 3 grams daily for 2 weeks. In Group II were 12 chronic urinary carriers who were positive for urinary schistosomiasis. These patients were given chloramphenicol at the same dosage level. Those who continued to excrete *Salmonella* organisms were then given treatment for schistosomiasis until they became negative for that infection. Individuals who still were persistent carriers were again treated with chloramphenicol. Group III consisted of 12 chronic urinary carriers who also were positive for urinary schistosomiasis. They were first given treatment for schistosomiasis until they became negative for that infection. Those who persisted as *Salmonella* carriers were then given chloramphenicol.

Group I patients were considered negative for urinary schistosomiasis when examination of at least three 24-hour urine samples were negative for ova. Patients in Groups II and III were discharging ova in the urine and almost all of them had a history of repeated infection during the past several years. The patients were all males between 17 and 45 years of age. In none of them was there clinical or bacteriological

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evidence of enteric fever at the time of admission or a history of fever within 6 months prior to admission. Urine specimens were obtained from the midstream with prior disinfection of the external genitalia. The urine was cultured on McConkey's medium for 24 hours and on S. S. (*Salmonella-Shigella* Agar Medium "Difco") for 48 hours. The organisms were then identified biochemically on ordinary sugar medium and typed serologically against stock sera. A patient was classified as a carrier if the same organism was obtained on 3 successive cultures, each 1 week apart, and he was considered cured if weekly cultures were negative for at least 6 weeks. Of the 36 patients studied 16 carried *S. typhi*, 16 *S. paratyphi* A, and 4 *S. paratyphi* B.

## RESULTS

Ten of the 12 patients in Group I ceased to excrete *Salmonella* organisms after chloramphenicol treatment. Two relapsed after 3 and 4 weeks respectively.

Of the 12 patients in Group II, 6 ceased to excrete *Salmonella* organisms after chloramphenicol treatment and 6 continued to be carriers. The latter 6 were then given treatment until they were negative for schistosomiasis but all continued to be carriers. However, when retreated with chloramphenicol they all ceased to excrete *Salmonella* organisms.

The 12 patients in Group III after treatment for schistosomiasis continued to be *Salmonella* carriers. When treated with chloramphenicol, after being negative for schistosomiasis, they all ceased to be carriers.

## DISCUSSION

Various regimens have been used in the treatment of *Salmonella* carriers. These included sulphadiazine, streptomycin, sulphadiazine plus streptomycin, sodium bicarbonate plus sulphadiazine plus streptomycin, and penicillin plus sulphamerazine. All these proved to be of little value.

With the marked success of chloramphenicol in the treatment of enteric fever it was only natural to try this drug's effect on chronic carriers. Using chloramphenicol, 4 grams daily for 12 to 14 days, Nichols[9] in 1950 treated six patients who had excreted *S. typhi* in the urine for 7 months to 2 years, and five of the six ceased to excrete organisms. Sporadic cases were treated successfully by Kennedy and Millar,[10] Nor El Din,[11] and Messih.[6] Miller and Floyd[4] in 1953 reported results in the treatment of chronic urinary *Salmonella* carriers in Egypt. Fifteen patients were given chloramphenicol, 30 grams in 10 days. Nine remained free for 1 year and five resumed excretion 5 to 22 days after treatment. When five were treated again for 20 days, two remained free for 1 year and three continued to excrete organisms. These 15 patients all had a history of schistosomiasis and showed ova in the urine.

The work reported here showed that chloramphenicol in a dosage of 3 grams daily for 2 weeks was effective in 10 of 12 patients who did not have schistosomiasis but gave favorable response in only 6 of 12 patients who had active urinary schistosomiasis. Treatment of *Salmonella* carriers by chloramphenicol apparently was less effective in the presence of urinary schistosomiasis.

In all of the 18 patients treated for urinary schistosomiasis and then given chloramphenicol for the carrier state either for the first or second time, the *Salmonella* organisms disappeared from the urine. These results confirm the observation that treating urinary schistosomiasis increases the response of carriers to chloramphenicol, and point out the important role of urinary schistosomiasis in the persistence of the carrier state.

The use of chloramphenicol in a dosage of 3 grams daily for 2 weeks and the repetition of this for a second course in some patients caused no adverse side effects. The hematological picture of all patients was followed closely during the study and no significant change was noted.

## SUMMARY

Thirty-six urinary *Salmonella* carriers were studied. Treatment by chloramphenicol was found less effective in the presence of urinary schistosomiasis. Treating urinary schistosomiasis was found to increase the response of carriers to chloramphenicol.

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# STUDIES ON *VIPERA PALESTINAE* VENOM. FRACTIONATION BY ION EXCHANGE CHROMATOGRAPHY*

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In a previous study[1] we described some toxicologic, biochemical and immunological properties of fractions of *Vipera* (*xanthina*) *palestinae* venom separated by paperelectrophoresis. The fractionation led to the discovery in this venom of two neurotoxins against which no antibody was present in the specific Pasteur horse antiserum hitherto used. By immunizing rabbits with the neurotoxic fractions following immunization with whole venom, an enriched antineurotoxin-containing serum with higher protective power was obtained.[2] Subsequently, immunization of horses by this procedure in the Pasteur Institute (Paris) led to the production of a potent antineurotoxin-containing antiserum[3] which is now available for clinical use.

The continuous paperelectrophoresis used for the preparation of larger amounts of *Vipera palestinae* venom fractions, however, is a cumbersome procedure. An endeavor was made, therefore, to separate the venom by ion exchange chromatography, a method applied recently by Boman and associates[4, 5] in the study of the venoms of the rattle snake *Crotalus adamanteus* and of the Ringhals cobra.[6] The use of a cation exchanger, Amberlite IRC-50-XE64, proved unsatisfactory as no sharp separation of active components was obtained and some, *e.g.*, the L-amino acid oxidase and the erythrocyte sphering factor,[7] were not recovered. On the other hand chromatography of the venom on the anion exchanger diethylaminoethyl (DEAE) cellulose proved a suitable method of fractionation, both as to resolving power and as to recovery of activities. Some properties of *V. palestinae* venom fractions, separated on DEAE cellulose, will be described in the present communication.

* This work was supported by the Sick Fund of the General Federation of Jewish Labour in Israel, and is part of a thesis submitted by C. Perlmutter to the Tel-Aviv University, in partial fulfillment of the requirements for M.Sc. degree in bacteriology.

## MATERIALS AND METHODS

*Venom.* Obtained from adult *V. palestinae* (milked in the Department of Zoology of the Tel-Aviv University), venom was dried from the frozen state and stored at −20°C. A single batch of 25 g dried venom collected over a period of 3 months from 100 snakes, was used throughout this investigation. A stock solution of 250 mg/ml in physiological saline was kept at −20°C in 1 ml portions, each being defrosted only once immediately before use. The $LD_{100}$ of this batch was 45 μg/20 g body weight. Venom solutions for chromatography were prepared by dialyzing the 25% venom stock solution against 3 changes of 100 volumes of the starting buffer. The amount of the solution applied to the column was corrected for volume increase due to dialysis.

### *Chromatographic Technic*

Diethylaminoethyl (DEAE) cellulose was obtained from Brown Co., Berlin, N.H. (type 20 Reagent Grad. Cap. 0.1 mEq./g).

Carboxymethyl (CM) cellulose was prepared according to Peterson and Sober[8] from Whatman ashless standard cellulose powder, mesh 325 and higher (H. Reeve Angel and Company Inc., New York).

The ion exchanger was sedimented in 50 times its volume of distilled water for 15 minutes and decanted to remove small particles. The sediment was mixed with 20 volumes of 1% NaOH and after 2 hours washed 10 times with large volumes of water and finally with Sorensen phosphate buffer of pH and molarity to be used as starting eluent. Subsequently the column was packed under overpressure of 0.5 atmosphere, using compressed nitrogen, and additional washings of buffer were passed until the pH of the effluent was identical with that of the eluent.

The dialyzed venom or venom fractions in phosphate buffer were applied to the column in small portions and followed by an additional

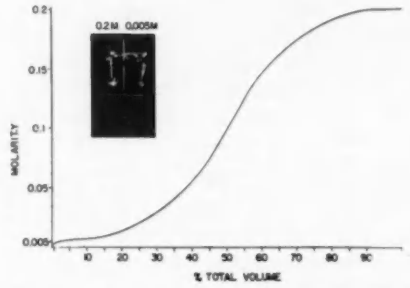


FIG. 1. Molarity of eluent obtained by mixing of two phosphate buffers in two-funnel system.

amount of starting buffer. Subsequently, gradient elution was carried out by using two interconnected separation funnels (Figure 1) containing phosphate buffers, 0.005 M and 0.2 M, respectively. The change in molarity of the resulting eluent is given in Figure 1. After completion of the gradient elution a final elution with 1 M NaCl was carried out. The amounts and pH of the solutions applied are specified in the experiments.

Effluent fractions were collected with Reco automatic fraction collector (Timer controller model F 1200 A. Research Equipment Corp., Oakland, California).

### *Analytical Methods*

*Protein content.* The optical density at 279 mμ, corresponding to maximal absorption for tyrosine, was determined. Control buffer served as blank.

*Toxicity.* Locally bred Swiss albino mice were injected intraperitoneally with 0.1–0.2 ml of venom fractions and their dilutions (in buffered saline pH 7.2), and observed for neurotoxic signs, as described previously,[1] time of death and peritoneal hemorrhages. The $LD_{100}$ (minimal dose lethal to all animals tested in one group), rather than the $LD_{50}$, was used as unit of toxic activity in order to economize venom fractions.

*Protease.* 0.05 ml of fractions or their dilutions were applied on undeveloped, fixed x-ray film. Following incubation at 37°C for 3 hours in a moist chamber, the film was washed with tapwater, immersed in Amido black 10 B solution for 30 seconds and then again washed with tapwater. Gelatin digestion was indicated by an unstained spot on a colored background.

*Amino acid oxidase.* L-ophio amino acid oxidase activity was estimated according to Boman and Kaletta.[5]

*Phospholipase A.* Hemolysis of human red blood cells in the presence of egg yolk was examined according to the procedure described by Boman and Kaletta;[5] hemoglobin liberation was judged by gross observation.

*Coagulation factors.* Standard procedures and systems, adding venom or fraction solutions in small amounts, were used for whole blood clotting time,[9] one stage plasma prothrombin time,[10] thromboplastin generation[11] and thrombin generation.[12] Plasma recalcification time: 0.1 ml oxalated human plasma (9 vol. blood + 1 vol. 0.1 M potassium oxalate) was mixed with 0.05 ml venom or fraction in buffered saline and 0.05 ml of 0.05 M $CaCl_2$ at 37°C. Thrombin-like activity: 0.1 ml human Cohn Fraction I, 2.2% in buffered saline, was mixed with 0.05 ml venom or fraction at 37°C and observed over 3 hours for appearance of a clot. For study of fibrinogen conversion-promoting action 0.1 ml thrombin solution, diluted to obtain suitable clotting times, was added to this system.

*Fibrinogenolysis and fibrinolysis.* 0.1 ml human Cohn fraction I, 2.2% in buffered saline, was mixed with 0.05 ml venom or fractions; 0.1 ml bovine thrombin solution (1000 U/ml) was added either immediately (clot observed for lysis during 3 hours at 37°C) or after 30 minutes incubation at 37°C.

*Erythrocyte sphering factor.* 0.05 ml venom fraction was added to 0.2 ml of a mixture of 1 part of whole oxalated human blood and 5 parts of a 2% polyvinylpyrolidone (PVP) solution in buffered saline, pH 7.2. The red cells were observed for sphering and inhibition of rouleaux formation. Packed red blood cells, washed 3 times and diluted 1:10 with PVP solution, to which venom fraction was added, served as control. It has been shown previously[7] that whole venom does not cause red blood cell disk-sphere transformation in the absence of plasma.

*Immunological tests.* Enriched specific horse antiserum (Pasteur) prepared by Kochwa, Izard, Boquet and Gitter[3] was used. The double gel diffusion method of Ouchterlony, modified by Uriel and Grabar,[13] was applied. Neutralization tests in mice were performed as described previously.[1, 2] Neutralization of in vitro activities of the various fractions was studied by incubating

them with antiserum in various proportions at 37°C for 1 hour and subsequent testing on the above substrates.

## RESULTS

### *Chromatography of* Vipera palestinae *Venom on DEAE-cellulose*

After equilibration with a starting phosphate buffer of pH 8.1, 0.005 M, 8 ml of dialyzed venom, corresponding to 4 ml 25% venom stock solution, was applied to the column (height 54 cm, diameter 1 cm) with a maximum velocity of 6 ml per hour, and followed by 100 ml starting buffer with velocity of 5 ml per 30 minutes. Gradient elution was started at the same velocity with 600 ml of phosphate buffer, pH 6.8 (300 ml of 0.005 M and 300 ml 0.2 M as indicated in "methods"). After 300 ml had been collected, the gradient elution was interrupted and 60 ml of phosphate buffer pH 6.8, 0.2 M was passed in order to avoid too large a spread of some fractions (observed in preliminary observations). Subsequently, 100 ml 1 M NaCl was passed. The eluate was collected in 5 ml samples.

Already during the development with the starting buffer two pigments could be distinguished, a violet pigment proceeding on the column and a yellow pigment remaining at the top. During the gradient elution the yellow pigment separated into two different shaded yellow pigments. The 560 ml effluent was collected in 112 tubes during a period of 56 hours. The protein content and the biological activities of the various fractions obtained are shown in Figure 2.

A first fraction, which had neurotoxic, anticoagulant, and proteolytic actions and contained a violet pigment and 10 antigens, appeared in tubes 9 to 19. The neurotoxin caused signs similar to the effect of fraction $K_1$-$A_1$ obtained by paperelectrophoresis in our previous study,[1] *i.e.*, paralysis of hind legs and respiratory death. The properties of the coagulant were identical with those described for whole venom.[14] The anticoagulant in this fraction, similar to the whole venom, prolonged the whole blood clotting time, the recalcification time, the Quick prothrombin time and inhibited thrombin generation and thromboplastin generation. It did not affect the clotting of plasma or of fibrinogen by thrombin.

A second principal fraction appeared in tubes

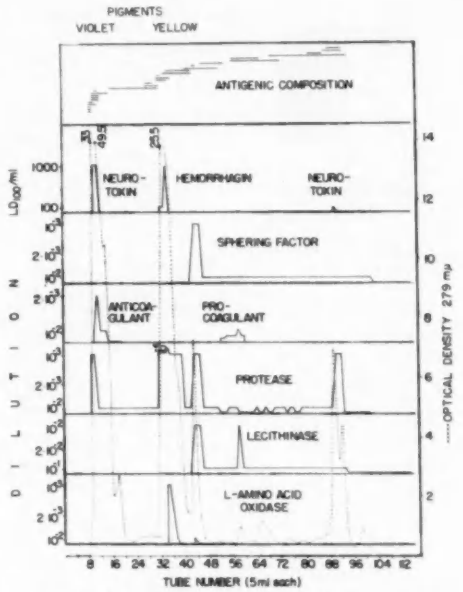


FIG. 2. Chromatogram of 1 g of *Vipera palestinae* venom on DEAE-cellulose. The dotted lines indicate the optical density at 279 mμ. The unbroken lines represent various activities, indicated in the respective horizontal compartments. The horizontal lines, representing the antigenic composition, show the spread of each antigen over the various fractions. The dilution figures on the ordinate represent the highest dilution at which the various activities were still discernible in the respective tests.

32 to 39 and had hemorrhagic and proteolytic actions and contained L-amino acid oxidase, a yellow pigment and 7 antigens.

A third fraction appeared in tubes 42 to 44 and contained a smaller amount of hemorrhagin, the red blood cell sphering factor, protease, lecithinase, L-amino acid oxidase, a second yellow pigment and 3 antigens.

The fourth fraction (tubes 57–60) contained weak hemorrhagic activity, a coagulation-accelerating factor, lecithinase and 2 antigens.

The impression was gained that the effect produced by the hemorrhagin in fraction 2 was different from that produced by fractions 3 and 4. The hemorrhagin in fraction 2 (tubes 23–40) caused wide spread hemorrhagic imbibition of the peritoneum, the diaphragm and of the muscular

layers of the abdominal wall; small blood clots and bloody fluid were found in the peritoneal cavity. The hemorrhagin contained in the third and fourth fractions (tubes 44 and 56) on the other hand caused less marked bloody infiltrations of the tissues; big clots were found in the peritoneal cavity, mainly around the splenic vein, and dilated and ruptured blood vessels were seen macroscopically.

The procoagulant shortened the recalcification time, the whole blood clotting time, the thrombin generation time, the thromboplastin generation time and the prothrombin time. It did not clot oxalated plasma in the absence of calcium; it did not clot fibrinogen (fraction I) but it accelerated the clotting of plasma and of fibrinogen by thrombin.

In between the third and fourth main fractions others could be distinguished with smaller protein content and having some sphering factor, protease, and lecithinase activity.

The fifth main fraction (tubes 88–93) had neurotoxic and protease activity and contained 4 antigens. The effect of the neurotoxin in this fraction was a generalized tremor similar to that of fractions $A_4$–$A_5$ obtained by paperelectrophoresis.[1]

A gel diffusion test with Pasteur antiserum (Figure 3) showed that there was no identity of antigens between the two neurotoxic fractions (fraction 1 and 5), or between the different fractions containing the hemorrhagins (fractions 2, 3 and 4).

The calculated total toxicity of the effluent, obtained by adding the amount of $LD_{100}$'s contained in all 5 ml samples of the toxic fractions, was 16775, of which 10650 was neurotoxic (paralytic neurotoxin 10050, tremor producing neurotoxin 600) and 6125 $LD_{100}$ hemorrhagic. As the 1 gram of whole venom applied to the column contained 22200 $LD_{100}$, the calculated recovery of lethal activity would be about 75%.

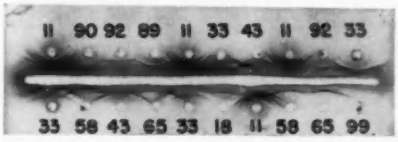


FIG. 3. Pasteur antiserum against *Vipera palestinae* venom fractions separated on DEAE-cellulose.

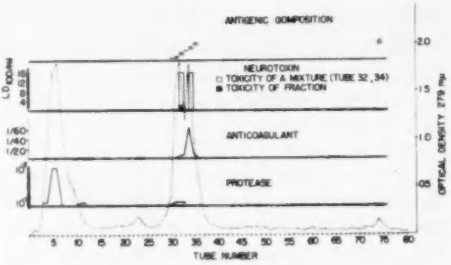


FIG. 4. Chromatography on CM-cellulose of neurotoxin-containing fraction (tubes 9–18, DEAE-cellulose effluent). For toxicity of neurotoxic mixture (tubes 32 and 34) see text.

*Rechromatography*

An attempt to fractionate the protein-rich paralytic neurotoxin-containing fraction which first passed the DEAE-cellulose column (tubes 9–18), was made by rechromatography on CM-cellulose. After equilibration with the starting buffer, pH 6.4, 0.005 M, a 10 ml mixture of 1 ml samples from each of the tubes 9 to 18, was applied to the column (15 × 1 cm) with a maximum velocity of 6 ml per hour and followed by 60 ml starting buffer at a velocity of 3 ml per 30 minutes. Gradient elution was performed with 300 ml phosphate buffer, pH 6.4 (150 ml 0.005 M and 150 ml 0.2 M). Subsequently, 30 ml 1 M NaCl was passed. The effluent was collected in 3 ml samples. The protein content and various activities of the fractions are shown in Figure 4.

The rechromatography resulted in separation of three main fractions. The first fraction (tubes 3–12) contained protease; the second had weak neurotoxic activity (4 $LD_{100}$/ml in tube 32) and little protease activity; the third contained the anticoagulant but was devoid of neurotoxic activity. When the second and third fractions were mixed in equal parts (tubes 32 and 34), the toxicity of the mixture was raised to 16 $LD_{100}$/ml, indicating a neurotoxin-potentiating action of the anticoagulant-containing fraction. Although the number of antigens found after rechromatography was identical with that of the fraction applied to the column, the recovery of neurotoxin was extremely poor (96 $LD_{100}$ out of 2000 $LD_{100}$, *i.e.*, about 5%). Because of this poor recovery final evaluation of the neurotoxin-potentiation phenomenon must be postponed.

*Neutralization by Specific Pasteur Horse-antiserum*

The Pasteur antiserum was able to neutralize both the paralytic neurotoxin of the first fraction and the tremor-producing neurotoxin of the fifth fraction in the DEAE-cellulose effluent, as well as the different hemorrhagins. Exact titrations were not carried out. The anticoagulant, the procoagulant and the protease activities of the various fractions were neutralized. The red blood cell sphering factor, the lecithinase and the L-amino acid oxidase activities on the other hand were not inhibited by the antiserum. It is noteworthy that the first protease fraction obtained by rechromatography on CM-cellulose, although its activity was inhibited by the antiserum, did not show a precipitation line in gel diffusion.

## DISCUSSION

Chromatography on DEAE cellulose has been proven an efficient method of fractionation of *Vipera palestinae* venom resulting in good separation of various toxins and enzymes. By this method, not only could known properties of the venom be attributed to different components, but, in addition, a biological activity-procoagulant not manifest in the whole venom could be detected. Although in the light of the recent studies of Björck and Boman[6] it cannot be excluded that chromatography in the cold might modify the pattern of separation, the results obtained seemed of sufficient interest to warrant presentation.

The calculated recovery of total lethal activity, 75%, was satisfactory and exceeded that obtained by the paperelectrophoretic method used in the previous study.[1] The efficiency of the separation is also evident in the recovery of a larger number of antigens than obtained by continuous paperelectrophoresis.[2]

The proportion of separate toxins obtained by chromatography on DEAE-cellulose is markedly different from that in paperstrip eluates. In the paperelectrophoretic procedure, the neurotoxic fractions constituted less than 20% of the total eluate toxicity, and the hemorrhagin more than 80%.[1] In the present method of preparation by ion exchange chromatography, neurotoxin was preponderant in the effluent, constituting more than 60% of the total toxicity while the hemorrhagin constituted about 35%. The significance of these findings is not yet clear. It is possible that by the different methods of fractionation various activities are being lost preferentially. The hemorrhages invariably present in mice following injection of *V. palestinae* venom, as well as in humans dying from the bite of this snake, seemed to point to the hemorrhagin being the determinative lethal agent. On the other hand it cannot be excluded as yet that the "neurotoxin" contributes greatly to the lethal action of the venom, *e.g.*, by action on the central nervous system, resulting in early death before development of neurotoxic signs such as paralysis or convulsions. The recognition of such a role of the neurotoxin in the lethal action of the whole venom would increase the significance of the therapeutic trial with antineurotoxin-containing serum,[3] now carried out systematically in Israel.

The presence of two different neurotoxins, indicated in the previous study,[1] one causing paralysis and the other generalized tremor, was confirmed. The paralytic neurotoxin, first passing the DEAE-cellulose column, had neurotoxic activity similar to whole venom neutralized with antihemorrhagic serum and to the paper-electrophoretic fractions $K_1$–$A_1$. The separation of this neurotoxin from lecithinase is in agreement with their independence in cobra venom.[15, 16] The results obtained by rechromatography on CM-cellulose of the paralytic neurotoxin-containing fraction showed that the bulk of the protease in this fraction has no neurotoxic activity, making the independence of these two activities very likely. Rechromatography showed even more clearly than the primary separation on DEAE-cellulose that the anticoagulant is separable from the neurotoxin as well.

The presence of different hemorrhagins, indicated by different chromatographic behavior, seems to be substantiated by non-identity of antigenicity and possibly by a difference in type of hemorrhage, caused by the second and the third fractions passing the DEAE-cellulose column. The pathogenesis of these hemorrhages is not clear. As has been pointed out in our previous study,[14] they are not caused by the anticoagulant which appears in a different fraction. Most probably they are related to protease activity, from which they could not be separated, and are due to vessel wall destruction.[17]

The detection of a procoagulant in *V. palestinae* venom, in addition to the venom's previously described anticoagulant activity, indicates that

this combination, hitherto known to occur in Russell viper venom,[18, 19] may be a more general feature of snake venoms. Whereas the procoagulant of Russell viper venom accelerates thrombin formation and its anticoagulant inhibits fibrinogen-fibrin conversion,[19] in the case of *V. palestinae* venom the procoagulant accelerates the latter reaction and the anticoagulant inhibits the former.[14] Preliminary observations have shown that in contradistinction to those venoms which clot fibrinogen in the absence of thrombin, *e.g.*, *Bothrops jararaca* and *Bothrops atrox*,[20, 21] the *V. palestinae* venom procoagulant accelerates thrombin-induced fibrin formation only. The procoagulant has not been separated from protease and a proteolytic mechanism of action cannot be excluded. It is pertinent, however, that neither the whole venom nor the separated procoagulant have fibrinogenolytic activities.

The separation of the anticoagulant from protease effected by rechromatography on CM-cellulose provides additional evidence for the previously proposed mechanism of antithromboplastic action of the venom,[14] namely by inhibitory action (reversible by antiserum) and not by destruction of thromboplastin.

The red blood cell sphering factor of the venom, producing non-lytic spherocytosis only after interaction with plasma,[7] was not separated from lecithinase by ion exchange chromatography. The exact relationship of the sphering factor to the lecithinase has not been clarified as yet and the possibility of the spherocytosis being induced by minute amounts of lysophospholipids formed by the action of the enzyme is under investigation.

Finally the association of the L-amino acid oxidase with the yellow pigment in two fractions of the DEAE cellulose effluent conforms with what is known on flavine adenine dinucleotide, constituting the prosthetic group of the enzyme.[22]

SUMMARY

*Vipera palestinae* venom was fractionated by chromatography on DEAE cellulose.

Two neurotoxic and three hemorrhagic fractions were obtained. The calculated recovery of total lethal activity was 75 per cent, about two-thirds of which was neurotoxic, one-third hemorrhagic.

Correlations of biological activities of the various venom components are discussed.

In addition to the known anticoagulant, a procoagulant accelerating the conversion of fibrinogen to fibrin in the presence of thrombin was found.

ACKNOWLEDGMENT

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# A QUANTITATIVE STUDY OF VENOM SECRETION BY *VIPERA PALAESTINAE*

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A comprehensive account and extensive review of literature on the subject of venom secretion was recently given by Klauber.[1] The main items considered by him are the total yield of venom and its concentration, the age and size of snakes in relation to venom secretion, and the influence of continuity of milking on venom yield. Klauber's review refers almost exclusively to various species of rattlesnakes (Crotalinae). Later studies on the subject have been reported by Reid[2] and Minton.[3] Theodor[4] gave a preliminary account of the venom yield of *Vipera palaestinae* based on information obtained from Dr. H. Mendelssohn.

It is well known that there are individual differences in the reaction of persons bitten by *V. palaestinae*, some suffering only local lesions, some having grave complications, while to others the bite is lethal. Reports of viper bite in Israel were analyzed by Hadar and Gitter,[5] who classified the victims into four groups according to the severity of the reactions. Among 303 cases reported during the period 1951 to 1953, when no antiserum was used, approximately 6% were fatal, 13% severe, 20% moderate, and 61% light. This variability in degree of injury may be due to differences in the victim's initial state of health, in the bite's location, or in the quantity and concentration of injected venom. In the present study an attempt was made to determine the individual differences in the general yield and concentration of venom; the quantity produced, stored and injected; the influence of temperature on the venom secretion and concentration; the relation, if any, between the venom yield and the age and size of the snake; the influence of sustained milking on the venom yield and concentration; and the relation between all these and the findings of Hadar and Gitter.[5]

Although all possible precautions were taken, it was not possible in all instances to obtain precise data. The work was performed with living, venomous snakes, which are difficult to handle. In most cases the entire content of the glands was collected, but occasionally some venom was lost, being ejected outside the container. Such incidents and others of the same nature must be taken into consideration. It is hoped that their influence has been overcome by the large number of experiments on which the conclusions are based.

## METHODS

About 150 *Vipera palaestinae* Werner 1938, of different ages and sizes, were available for study: seven had recently hatched and were not yet molted; 15 were juveniles, less than 1 year old; 11 were of medium size, about 1 to 2 years old; and all others were adults of different sizes and of unknown age.

The methods used, with minor modifications, were those introduced by Dr. H. Mendelssohn. No attempt was made to keep the snakes under simulated natural conditions. They were housed in a room 8 meters by 3 meters and kept in separate cages.

The snakes were provided with water at all times and were offered two white mice of known weight twice weekly at night. They accepted, generally, less food than was offered, sometimes without apparent cause but more often because of molting or prevailing low temperatures. If heat was not provided, feeding stopped in October or November and started again in April or May.

Extraction of venom was done in the manner described by Klauber,[1] except that no hook was used. By this method practically all venom was extracted, at least all from the lumen of the venom gland. Additional pressure, however strong, would not produce more venom. Mice bitten by a snake immediately after its milking remained alive.

The vipers were milked, in most cases, into separate vials, the venom being dried over calcium chloride.

Some snakes were heated in winter on electric heating plates, which kept the cage temperature at a range of 30 to 35°C; but, as each snake was allowed to select its place inside the cage, there was no possibility of determining either the exact

temperature to which a snake was exposed or its body temperature.[6-8]

## RESULTS

Twenty-two adult snakes were milked during three consecutive seasons, the summers of 1957, '58 and '59. Some of them had also been milked previously. They were divided into groups of 8, 6, and 8, designated A, B, and C, and during the summer of 1957 the three groups were milked at intervals of 4, 3, and 2 weeks respectively. In the winter of 1957–58 the 6 snakes of Group B were artificially heated and milked at intervals of 4 weeks; they were continued on this schedule without interruption at all seasons. For A and C groups the intervals between milkings were mutually interchanged during the summer of 1958, and in the summer of 1959 all groups were milked at one-month intervals.

Of the original 22 vipers, 16 died at different times during the course of the experiments. This seemingly rather high rate of mortality was caused, at least partly, by the continuous milking to which the snakes were subjected.

Altogether 300 samples of venom were obtained, each derived from a separate milking. The separate yields varied considerably. This intra-group variability tended to obscure group differences which could have been ascribed to the different intervals between milkings. Such inter-group differences as have been recorded proved to be statistically insignificant (Table 1). As noted by others,[1, 9, 10] differences were also found in the amount and concentration of venom obtained from individual snakes at successive milkings.

Before turning to the actual venom-yields obtained, it should be stated that the $LD_{100}$ and $LD_{50}$ amounts of whole *Vipera palaestinae* venom for white mice of 15 g body-weight are 0.055 mg and 0.038 mg respectively.[11]

TABLE 1

*Average yields of venom from 22 vipers milked at intervals of 2, 3 and 4 weeks during summer 1957*

| Intervals between milkings | Group | Average yield per snake, per milking | | |
|---|---|---|---|---|
| | | Fresh, mg | Dry, mg | Conc., % |
| 2 weeks | C | 253 | 57 | 22.5 |
| 3 weeks | B | 266 | 60 | 22.5 |
| 4 weeks | A | 290 | 68 | 23.4 |

### *The Influence of Some Factors on Venom Secretion*

*Continuity of milking.* Klauber[1] found a decline in venom production of snakes kept in captivity, but he thought that good care and ample food might improve the yield. From figures given by him for *Crotalus ruber lucasensis* it seems, however, that when these snakes were given an adequate period of rest between successive milkings, the decrease in yield was unimportant. Minton[3] found a progressive increase in the venom yield from one individual *Crotalus atrox*. This, however, was a young snake, which during the period of investigation grew to twice its original length. Wolff and Githens[12] found no decrease in the yield, concentration, or toxicity of venom from 22 *Agkistrodon piscivorus* milked over a period of 2 years. Their snakes, however, were not milked at regular intervals and the prevailing temperatures were not stated.

As already mentioned, our snakes were milked during 3 years, some of them at all seasons. The amount of venom extracted from each animal tended to *increase* from season to season (Table 2). Some of these snakes upon reaching their 30th milking had yielded more than 2000 mg of dry venom per individual (Table 4).

The body weights of the snakes given in Table 3 show that the increase in venom secretion cannot be ascribed to weight gain alone. In one in-

TABLE 2

*Average yields of venom of 5 regularly-milked vipers (groups A and C), obtained during July–November 1957 and July–November 1958*

| No. of snake | Group | Year | Interval between milkings, weeks | Average yield per milking | | |
|---|---|---|---|---|---|---|
| | | | | Fresh, mg | Dry, mg | Concentration, % |
| 8 | A | 1957 | 4 | 361 | 81 | 22.4 |
| | | 1958 | 2 | 400 | 84 | 21.0 |
| 12 | A | 1957 | 4 | 199 | 46 | 23.1 |
| | | 1958 | 2 | 380 | 76 | 20.0 |
| 16 | A | 1957 | 4 | 258 | 59 | 22.9 |
| | | 1958 | 2 | 289 | 57 | 19.7 |
| 7 | C | 1957 | 2 | 281 | 61 | 21.7 |
| | | 1958 | 4 | 444 | 98 | 22.1 |
| 9 | C | 1957* | 2 | 304 | 67 | 21.8 |
| | | 1958 | 4 | 465 | 98 | 21.1 |

* Milked July–September only, because of a wound on the neck.

TABLE 3

*Maximal quantities of venom obtained at a single milking from 5 vipers (groups A and C) during 3 years, 1957, 1958 and 1959*

| No. of snake | Group | Date of milking | Weight of snake, g | | Length of snake, mm | Maximal yield, mg | | |
|---|---|---|---|---|---|---|---|---|
| | | | Spring | Autumn | | Fresh | Dry | Conc. % |
| 8 | A | 13.IX.1957 | 673 | 562 | | 500 | 108 | 21.6 |
| | | 17.VII.1958 | 595 | 643 | | 604 | 141 | 23.3 |
| | | 11.IX.1959 | 674 | | 1100 | 990 | 217 | 21.9 |
| 12 | A | 19.VII.1957 | 561 | 490 | | 288 | 69 | 23.9 |
| | | 25.IX.1958 | 472 | 598 | | 536 | 114 | 21.2 |
| | | 16.VII.1959 | 591 | | 1020 | 491 | 103 | 21.0 |
| 16* | A | 8.XI.1957 | 344 | 550 | | 399 | 83 | 20.8 |
| | | 11.IX.1958 | 516 | 583 | 990 | 455 | 101 | 22.2 |
| 7 | C | 11.X.1957 | 574 | 571 | | 407 | 89 | 21.8 |
| | | 28.VIII.1958 | 552 | 678 | | 523 | 115 | 21.9 |
| | | 10.VI.1959 | 706 | | 1080 | 495 | 111 | 22.4 |
| 9 | C | 13.IX.1957 | 643 | 495 | | 438 | 93 | 21.2 |
| | | 28.VIII.1958 | 455 | 428 | | 665 | 142 | 21.3 |
| | | 10.VI.1959 | 310 | | 1050 | 607 | 119 | 19.6 |

* Died in winter 1959.

stance, at least, the weight of a snake was reduced considerably (No. 9) and the venom yield, on the other hand, increased. Although there was a small increase in average prevailing temperatures during the second season, summer of 1958, it is not likely that the increase of venom yield can be ascribed to this factor.

The fact that a long period of milking does not impair the secretion of venom is perhaps even more evident when the yield from captive snakes is compared with that derived from freshly caught individuals milked for the first time. The latter gave in most cases amounts of only 200 to 400 mg of fresh venom. However, appreciable amounts of venom presumably were lost by some of the freshly caught vipers during their capture.[1]

*Temperature.* The maximum yields of venom were obtained during the hottest months of the summer, whereas in spring and late autumn the amounts decreased markedly. In winter no venom was produced at all. Snakes which were milked in December and again in May yielded very little venom (about 20 mg dry) and even this had probably been produced in the spring, a short time before milking. Figures 1 and 2 show the relation between the amount of venom extracted and the average minimum and maximum temperatures prevailing during the intervals between milkings as recorded in the serpentarium. Temperature also had an evident influence on the venom con-

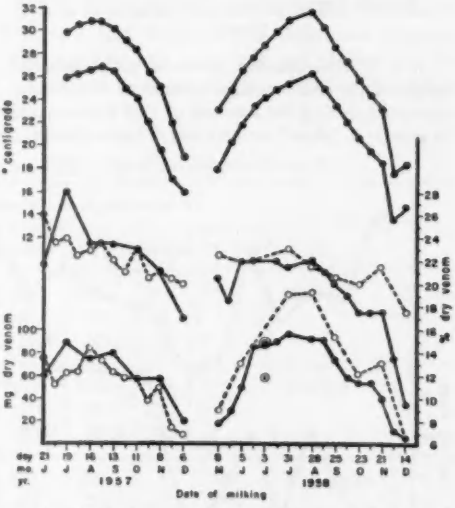


FIG. 1. Influence of temperature on yield and concentration of venom in experimental groups A and C during two milking seasons, June to December 1957 and May to December 1958. The minimal and maximal temperatures were computed as averages of the period preceding the milking. Points off the yield curves indicate that venom was ejected outside the milking vial. Group A (solid line) consisted of seven snakes in 1957, three in 1958; in group C (broken line) there were four snakes in 1957, two in 1958.

centration. Relatively more dry material was obtained at high than at low prevailing temperatures. However, the first milking in spring, immediately after hibernation, yielded a rather concentrated venom, presumably because of reabsorption of water during the long period venom was stored in the glands. The concentration of the venom extracted from vipers *collected* in winter lies within the usual range.

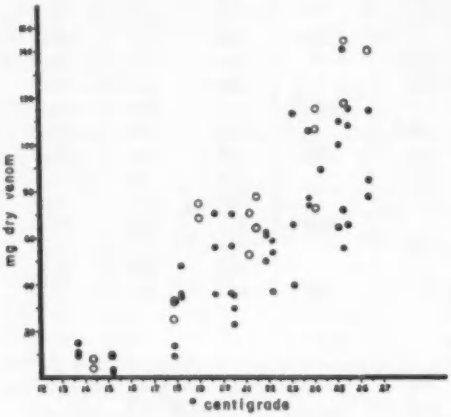


FIG. 2. Relationship between daily minimal temperature and average amount of dry venom extracted during the summer of 1958 from snakes in groups A (closed circles) and C (open circles).

The influence of temperature is even more evident in the heated group of vipers. Figure 3 shows the usual rise of venom yield during the summer months and its decline at the approach of the cold season. At that juncture the heat was turned on with a consequent immediate increase in venom yields. During the period of artificial heating larger amounts of venom were obtained than at any other time; this made it possible to collect considerable quantities of venom in a relatively short time (Table 4). From one of these heated snakes (No. 19) the highest total amount of venom was obtained, over 2000 mg dry material in 19 milkings. A similar quantity was also extracted from the non-heated snake No. 8, but in 30 milkings.

These results are laboratory findings and cannot, of course, be assumed to apply to conditions in the field. However, vipers freshly collected in summer and autumn gave higher yields of venom than those caught in spring. The average yield of fresh venom from 24 snakes acquired during April to May 1958 was 220 mg (43 mg dry), the concentration being 19.5%. Fifteen snakes collected in August to November 1958 gave on the average 395 mg fresh venom (85 mg dry) per

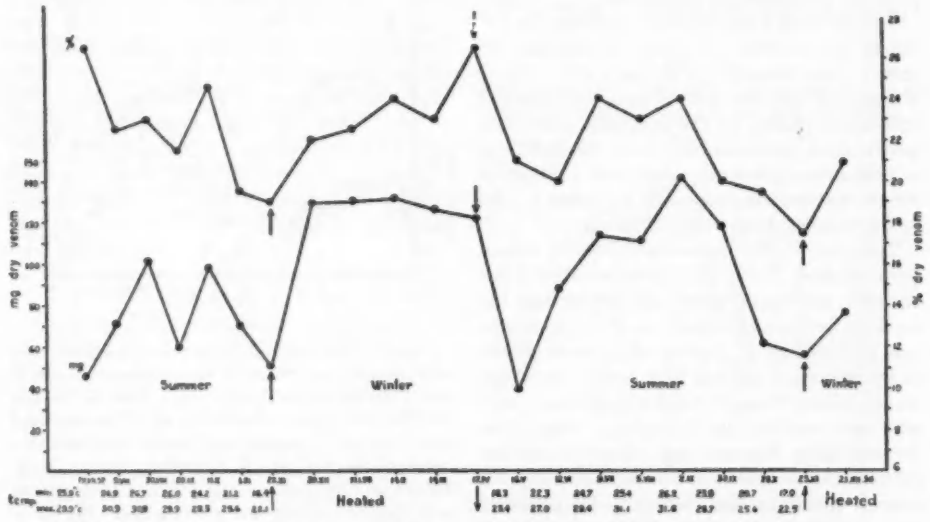


FIG. 3. Curve of yield and concentration of venom from snake No. 21 of group B, heated during the winter.

snake with a concentration of 21.5%. Vipers collected during the winter months had considerable amounts of stored venom, probably derived from secretion during the preceding summer season.

*Frequency of milking.* In venomous snakes killed 24 hours after milking, Minton[13] found a small amount of venom, 0.05 ml (about 50 mg), but there is no mention of the season, or of the prevailing temperatures. Klauber[1] found a slow rate of venom restoration. Parrish and Thompson[14] reported moderate venenation in 3 humans bitten by two species of *Crotalus* 24 to 28 hours after milking.

TABLE 4

*Total productivity of vipers artificially heated during the cold season (group B) and milked at intervals of 3 weeks in 1957, and monthly in 1958 and 1959*

| No. of snake | Duration of experiment, months | Number of milkings | Total yield, mg dry venom | Remarks |
|---|---|---|---|---|
| 17 | 5 | 7 | 363 | Died |
| 18 | 26 | 25 | 1565* | Milking continued |
| 19 | 17 | 19 | 2070 | Died |
| 20 | 9 | 11 | 982 | Died |
| 21 | 19 | 21 | 1949 | Died |
| 22 | 26 | 25 | 1822* | Milking continued |

* Total yields were slightly higher, three milkings during the winter of 1958–9 having been omitted.

For the purpose of studying the rate of venom regeneration, five series of experiments were conducted involving 61 vipers not including the 22 of the three experimental groups mentioned above. These vipers were milked (collectively, for each series) at different intervals, beginning at 1 day and extending up to intervals of 16 days. The results are presented in Table 5 and Figure 4. The rise in yield of fresh venom was rapid during the first week; subsequently production was much slower, so that individual differences became evident.

About 150 samples were taken from the snakes referred to above as A, B, and C groups milked individually at intervals of 4, 3 and 2 weeks during the summer of 1957. As can be seen in Table 1 and Figure 5 the differences between these groups are very small and statistically insignificant because of the notable individual

TABLE 5

*Rate of regeneration of venom indicated by yields after different periods of rest*

| No. of snakes | | Average yield of venom per snake after a rest of | | | | | | | Remarks |
|---|---|---|---|---|---|---|---|---|---|
| | | 1 day | 2 days | 3 days | 4 days | 7 days | 14 days | 15 days | |
| 15 | Fresh, mg | 110 | 157 | | 252 | 308 | 394 | | Heated |
| | Dry, mg | 18 | 29 | | 49 | 62 | 93 | | |
| | Conc., % | 16.3 | 18.4 | | 19.4 | 20.1 | 23.6 | | |
| 12 | Fresh, mg | | 135 | | 205 | 233 | 343 | | Heated |
| | Dry, mg | | 27 | | 41 | 49 | 77 | | |
| | Conc., % | | 20.0 | | 20.0 | 21.0 | 22.4 | | |
| 10 | Fresh, mg | 67 | 115 | | 230 | 360 | | 451 | Not heated |
| | Dry, mg | 10 | 19 | | 43 | 74 | | 97 | |
| | Conc., % | 14.9 | 16.5 | | 18.6 | 20.5 | | 21.5 | |
| 13 | Fresh, mg | 63 | | 130 | | | | 329 | Not heated |
| | Dry, mg | 10 | | 22 | | | | 74 | |
| | Conc., % | 15.8 | | 16.9 | | | | 22.5 | |
| 11 | Fresh, mg | | 46 | | | 114 | | | Medium-sized (Sub-adults) Not heated |
| | Dry, mg | | 8 | | | 24 | | | |
| | Conc., % | | 17.3 | | | 21.0 | | | |

variability. In the following season (summer 1958) when the intervals between milking of group C (2 weeks) and those of group A (4 weeks), were interchanged, somewhat clearer results were obtained. Whereas the increase in venom yield of group C when milked once every 4 weeks was very large, only a small corresponding rise could be observed in group A (Tables 1 and 2). When data of Table 2 are compared with those of Table 5, a higher yield of venom is seen after an interval of 1 week than after 2 and 4 week intervals. This apparent paradox is explained by the fact that the results of Table 5 were obtained during summer (in order to exclude the factor of temperature) whereas the data of Table 2 are averages of milkings during July to November, thus including low autumn yields. The change observed in the concentration of venom at short milking intervals is of special interest (Table 5, Figure 4). The amount of dry venom was very low after a short period of rest, rising with the increased length of intervals between milkings. This leads to the assumption that the venom when first secreted is dilute and that during storage in the glands it is concentrated, presumably by reabsorption of water.

The differences in yield and concentrations among the series milked at short intervals merit attention (Table 5). In spite of the fact that the experiments were performed during the summer, there was a higher total yield and concentration in the artificially heated snakes. These snakes secreted more venom and concentrated it faster. Somewhat similar results were obtained from the medium-sized snakes, because of their higher rate of metabolism. Especially obvious in this group

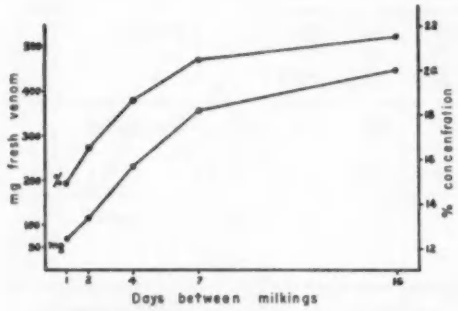


FIG. 4. Yields of fresh venom and concentration at different intervals between milkings, July to August 1958.

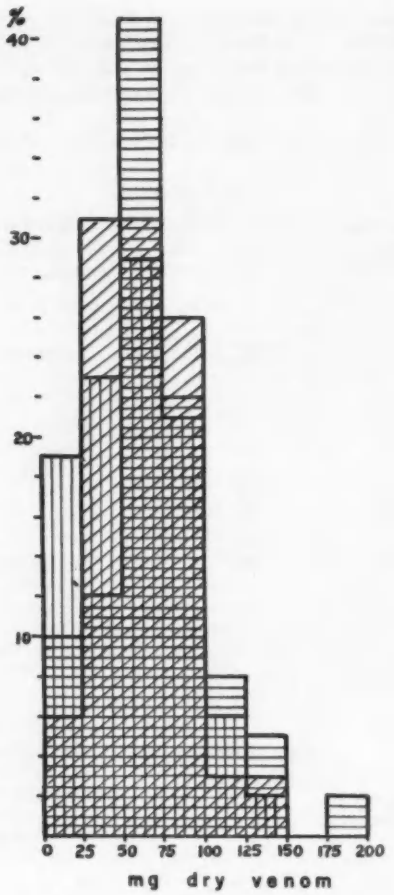


FIG. 5. Overlapping histograms of the average dry venom yields from groups A, B and C, milked at intervals of 4, 3 and 2 weeks respectively, during summer 1957. The frequencies are expressed in per cent of the total number of milkings. Group A (horizontal lines) was milked 41 times at intervals of 4 weeks; group B (diagonal lines), 35 times at intervals of 3 weeks; and group C (vertical lines), 48 times at intervals of 2 weeks.

was the great yield after 7 days as compared with the yield after 2 days of rest. These snakes also concentrated their venom more rapidly. Factors such as molt, pregnancy or food consumption were found not to influence the amount of venom secretion, nor its concentration. In unreported studies on adult vipers no relation could be found

between the size (expressed in terms of body weight) and the quantity of venom secreted.

*Injection of Venom*

In an attempt to find the quantity of venom injected Acton and Knowles[15, 16] adopted a method by which both snake and rat were held firmly, so that there was not a natural bite but rather a milking of the snake into the leg of the rat. In this way they obtained very high results from the first strike (more than 59% of the venom available in the glands) and very low results from the second bite.

The present experiments were made with a series of 20 snakes, some of which were used in other experiments reported in this paper. Each snake was offered a freshly killed mouse of about 15 g weight. Mice were chosen for these experiments in preference to artificial contraptions such as membranes, balls or watch glasses.[9, 17] Results obtained from tests with animal tissue with its specific properties, such as elasticity and ease of penetration, probably give a better approximation of the snake bite in nature, as mice, rats and other small animals are the usual food of *Vipera palaestinae*. Such tests also may give a better indication of the amount of venom injected into humans.

The mice were killed before the experiment by a blow on the head. Dead mice were used in order to prevent loss of weight by excretions, salivation, breathing, etc. The only remaining cause of decrease in weight was loss of moisture through evaporation during the interval between weighing and the injection of venom. This loss, though small, was taken into consideration. Each dead mouse was kept and weighed in a closed weighing vial, removed with forceps and introduced into the cage of a viper. The mouse was held by the tail with the forceps and kept clear of the walls and floor of the cage in order to prevent any unnecessary loss or gain of weight. After being stricken, the mouse was returned and the vial weighed, the amount of venom injected thus being established. After completion of a series of strikes, each snake was milked and the yield, fresh and desiccated, weighed. This made it possible to calculate the proportionate amount of venom injected at each strike in relation to the total venom available in the glands prior to each strike. All experiments were performed during the summer, in order to equalize the influence of the temperature

TABLE 6

*The quantity of venom injected by three different snakes in successive bites and its relation to the available (stored) quantity prior to each bite*

| | Amount injected | | | | | |
|---|---|---|---|---|---|---|
| Snake..... | 140 | | 19 | | 22 | |
| No. of strike | mg | % | mg | % | mg | % |
| 1 | 9.4 | 3.9 | nil | | 30.0 | 7.0 |
| 2 | 26.8 | 11.7 | nil | | 190.4 | 48.0 |
| 3 | 23.6 | 11.6 | 64.4 | 7.0 | 73.0 | 35.4 |
| 4 | 36.4 | 20.9 | 9.2 | 1.0 | 6.2 | 4.6 |
| 5 | 13.4 | 9.4 | nil | | nil | |
| 6 | 27.8 | 21.6 | nil | | 2.8 | 2.2 |
| 7 | 17.0 | 16.8 | nil | | 14.6 | 11.7 |
| 8 | 14.6 | 17.4 | nil | | 48.2 | 44.0 |
| 9 | 29.4 | 42.7 | 35.4 | 4.3 | 34.0 | 55.5 |
| 10 | 9.0 | 24.3 | 23.6 | 2.9 | 3.4 | 12.5 |
| 11 | 2.8 | 10.0 | | | 9.8 | 41.1 |
| 12 | 7.4 | 29.3 | | | 9.6 | 68.5 |
| 13 | 2.0 | 11.2 | | | 4.4 | |
| 14 & 15 | 4.8 | | | | | |
| 16 & 17 | 4.4 | | | | | |
| 18 & 19 | 3.6 | | | | | |
| 20, 21 & 22 | 5.8 | | | | | |
| 23 | nil | | | | | |
| Total injected | 238.2 | | 132.6 | | 426.4 | |
| Milking | nil | | 784.6 | | nil | |
| Total available | 238.2 | | 917.2 | | 426.4 | |

factor. No differences were found between the strikes of calm or excited snakes or between a spontaneous strike and a forced one. No distinction was made between a strike, a bite or a stab.[1]

The variability in the quantities of venom injected could be observed among the different snakes, in a series of strikes of the same snake, and also in the proportionate amount injected out of the total quantity available. Table 6 shows an "ideal" experiment with a snake (No. 140) which was always ready to strike. It spent the whole quantity of venom available in its glands; nothing more could be pressed out by milking. This shows that a snake is able to empty its glands in a series of bites, by the pressure applied by its own muscles. The same table also shows the variations between the different injections and the results of two further experiments carried out with other snakes. In most strikes a quantity

of less than 50 mg fresh (about 11 mg dry) venom was injected, with a mean of 32.1 ± 3.1. The proportionate amount injected was in most cases less than 15% of that available in the glands (mean 11.4 ± 1.0%).

Some vipers refused to cooperate from the very beginning, and others volunteered a few strikes, or only one, even though their glands were full of venom. At times vipers did strike, but without detectable injection of venom. Of 97 strikes (including only the first ten) 21 were blank. The snakes which delivered these nonvenomous bites proved to have considerable quantities of venom available in their glands (Table 6), showing that blank bites are caused by the malfunction of the injecting mechanism and not by the absence of venom as might be assumed.[3, 4] On the other extreme, there were injections of very large amounts of venom, up to 48% of the total content of the venom glands, and individual snakes occasionally injected unusually large amounts of venom following one or more blank strikes (Table 6).

## DISCUSSION

The methods employed made it possible to milk individual snakes on more than 30 separate occasions during a 3 year period, and to extract large quantities of venom from each snake. The frequency most practical for milking *V. palaestinae* seems to be once a month. A given quantity of venom can be obtained more quickly by milking the snakes at intervals of 2 weeks, but this seems to increase the mortality rate. It seems to be better not to extract venom from snakes in ecdysis as this may cause lesions on the neck. It is desirable to allow the vipers a period of rest in winter; but, if necessary, they may be heated and large amounts of venom extracted in a comparatively short time.

The concentration of venom is individually variable to some extent and is increased by higher temperatures and longer periods of rest between milkings. Most samples yielded between 20 and 23% dry venom, but higher and lower figures were recorded, which could not be ascribed to the influence of temperature or the intervals between milkings. Some snakes yielded extremely high or extremely low concentrations of venom, the range being from more than 50% dry venom to less than 10%.

The main question which arises is how these findings compare with what actually occurs in nature. The freshly collected vipers each gave at the first milking 200 to 400 mg of fresh venom with concentrations of about 20%. These are lower figures than were obtained in the laboratory with "trained" and heated snakes. The newly caught snakes show the same tendencies, however, as regards the influence of the temperature factor. Those collected in spring gave a smaller yield than those collected in summer and autumn, contrary to Klauber's[1] findings in *Crotalus viridis viridis*. Nothing definite can be stated about the exact temperature range to which *Vipera palaestinae* is exposed in the field where it has free access to a micro-climatic environment of its own choice.[6-8]

Snakes collected in winter have in their glands a considerable quantity of venom, probably secreted during the preceding summer and stored during the entire time elapsed since then. The popular belief that a snake is less dangerous in winter than in summer may be true only because of the sluggishness of the snake in winter, not because of the somewhat lower content of the venom glands.

With regard to the injection of venom it should be emphasized that a snake injects only a small amount of the total content of its glands when striking, using its venom economically. The second strike generally gives about the same amount of venom as the first, and contrary to findings by Acton and Knowles,[15, 16] as much or more venom may be injected in a subsequent bite, the amount decreasing only after the venom glands have been partially emptied. These findings contradict the general belief that a snake is less dangerous after its meal or after a few successive strikes.

In about 20% of the cases the snakes performed blank bites, *i.e.*, bites without any detectable injection of venom. These cases can be interpreted in the light of the detailed mechanics of the venom apparatus.[18]

In conclusion, the fate of a person bitten by a viper is decided not only by his own state of health, by the location of the bite and by the medical treatment rendered to him, but also by the amount of venom stored at the time in the glands of the snake, by the venom's concentration and by the functioning (or malfunctioning) of the venom apparatus, which determines the amount of venom injected. The intraspecific variability in the toxicity of the venom may also have some importance.[1, 19]

The variability in the doses of venom injected

may be roughly compared with the results obtained by Hadar and Gitter[5] in their study of the severity of symptoms in human victims bitten by *Vipera palaestinae* and not treated with antivenom. The fatalities represented 6.3% of all cases, a percentage similar to that of strikes with injections of more than 75 mg of fresh venom observed in the present study. Likewise, the light cases represented 61% of all cases, the same frequency as injections of less than 40 mg fresh venom. The figure of 75 mg fresh venom may perhaps represent a crude approximation of the minimal lethal dose for human beings. This method of estimating MLD for humans is not claimed to be accurate, but seems to be the only reasonable one available. In any case, it is more plausible than the estimate obtained extrapolating the MLD experimentally obtained for mice to arrive at the MLD of humans, such figures being higher than the total maximum yield of venom.[4]

It is well understood that the above comparison between laboratory results and actual cases of snake bites in nature is rather crude and involves some speculation. It is based on a rather small number of experiments and does not take into account the location of the bite, the state of health of the person bitten, the size of the snake, the season, or the number of bites inflicted. In spite of these reservations, the figures obtained and the comparisons made in the preceding paragraphs may have some practical bearing on the future dosage adopted in the clinical routine of snake bite treatment.


## SUMMARY

Observations on the secretion of venom by 150 captive *Vipera palaestinae* indicate that snakes separately caged and properly cared for may be milked regularly for 3 years or longer, and large quantities of venom can thus be obtained. With artificial heating, the milking can be continued throughout the year. No influence of ecdysis, pregnancy, or food consumption on the yield of venom was observed. Taking into account the amounts and concentration of the venom obtained as well as the physical well being of the snakes, a period of rest of one month between milkings is suggested. Temperature was found to be one of the factors governing the venom yield. A rise in temperature increased both the amount and the concentration of venom. The freshly secreted venom is rather dilute initially, and is subsequently concentrated by the reabsorption of water.

The amount of venom injected in a single bite was estimated by allowing the vipers to strike dead mice. The amount injected was found to range from nil to 190 mg of fresh venom; in most bites less than 50 mg amounts (mean, 32 ± 3.1 mg) were injected. The mean proportionate amount injected was approximately 11% of the venom available in the glands; in most strikes less than 15% of the available venom was injected. Results obtained in the laboratory when considered in relation to the severity of viper bites in human beings suggest that the minimal lethal dose of *Vipera palaestinae* venom for man is approximately 75 mg.



## ACKNOWLEDGMENTS

The present investigations were made during 1956 to 1959 at the Serpentarium of the Experimental Zoo of the Department of Zoology, Tel-Aviv University, within the scope of a project of venom extraction for the preparation of a specific antiserum against the bite of the local viper, *Vipera palaestinae* Werner 1938. This project was sponsored by the Ministry of Health, Government of Israel.

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# STUDIES ON THE VENOM OF *ECHIS COLORATA**

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*Echis colorata* Günther 1878, one of the Viperidae found in Israel and other Near East countries, is a thin, sand colored snake, up to 80 cm long, active during the night. It is found in stony regions, more especially in the Negev desert and in the Dead Sea region, but not in the sandy coastal area. Whereas *Vipera palestinae*, the venomous snake inhabiting the densely populated coastal area, has been responsible for the great majority of snake bite in Israel until the present, the danger of bite from *E. colorata* and other venomous desert snakes such as *Aspis cerastes* and *Walterinnesia aegyptiae* is increasing with the extension of settlement into hitherto uninhabited desert areas.

A detailed description of the characteristics, distribution and biology of *E. colorata* has been given by Theodor[1] while Shulov *et al.*[2] published data on the toxicity of its venom by the subcutaneous route in mice: $LD_{50}$ 77.5 μg/15 g body weight, restlessness, redness and swelling, hemorrhagic blisters followed by necrosis at the site of injection, finally paralysis of limbs and death without convulsions.

No cases of snakebite by *E. colorata* in the human have been reported to our knowledge so far. On the other hand clinical descriptions and toxicological and immunological studies have been reported for the African and Indian *Echis carinata*[3-8] which, according to Chopra and coworkers,[6] while rather different from all other Indian venomous snakes, is closely related to *E. colorata*.

The following study on *E. colorata* venom is based on methods recently applied in our laboratory[9-13] in the study of *V. palestinae* venom. Toxicological and biochemical studies of the whole venom and of its fractions separated by ion exchange chromatography were carried out, and a specific rabbit antiserum was prepared.

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## MATERIALS AND METHODS

*Venom.* The turbid pale yellow venom obtained from adult *E. colorata* (kept in the serpentarium of the Department of Zoology of the Tel-Aviv University) was dried from the frozen state and stored at −20°C. The light yellow powder constituted about 20% (w/w) of the fresh venom. A pool of 4 different batches of venom (400–500 mg dry weight each), collected from 12 snakes monthly for 3 months during the summer season, was used throughout this investigation. Stock solutions of the venom in physiologic saline 100 mg/ml were kept at −20°C in small portions and defrosted immediately before use in the experiments.

*Analytical methods.* The technic of chromatography on diethylaminoethyl (DEAE) cellulose and the methods for determination of protein content of fractions, toxicity, protease, amino acid oxidase, phospholipase A, erythrocyte sphering factor, coagulation factors, fibrinogenolysin and fibrinolysin and immunological tests were as described in a previous study on the venom of *V. palestinae*.[13] Immunoelectrophoresis was performed according to Uriel and Grabar.[14] Hyaluronidase was measured by the turbidimetric assay of Tolksdorf.[15] Plasminogen deficient fibrin clots, required for the study of the mechanism of action of the fibrinolysin, were prepared according to Alkjaersig *et al.*[16]

*Preparation of antivenin.* Two rabbits weighing 2 kg each were injected subcutaneously between the scapulae once a week for 6 weeks with increasing amounts of *E. colorata* venom solution (1–5 mg dried venom) in a 1:1 blended mixture with adjuvants (Bajol F + Arlacel A 9:1).[12] The rabbits were bled by heart puncture. Pasteur anti-*V. palestinae* horse serum (containing anti-neurotoxin)[17] was used for cross immunity experiments.

## RESULTS

### *Whole Venom*

*Toxicity.* The toxicity of the venom and of venom fractions was studied in locally bred albino mice by the intraperitoneal and intravenous routes.

*Intraperitoneal injection.* The $LD_{50}$ by the intraperitoneal route was 31 μg per 20 g mouse, whereas 36 μg or more was lethal to all the mice. Immediately after the injection of a lethal dose the animals showed restlessness followed by immobility, difficult breathing and general weakness. Preterminally paresis of the hind legs and jerky, uncoordinated movements of the forepaws were observed. The time of death depended on the dose of venom injected, 1 $LD_{50}$ causing death after 24 to 48 hours, 3 $LD_{50}$ in 45 to 90 minutes. Examination of the blood obtained from the heart of two mice, sacrificed 2 hours after the injection of 2 $LD_{50}$, showed a severe anemia (4 and 5 g %, preinjection tail blood 12 and 15 g %), severe thrombocytopenia (48,000 and 100,000 per cmm, preinjection tail blood 400,000 and 1000,000 per cmm), no change in white cell count, incoagulability of the blood (30 minutes, normal clotting time 1–2 minutes) and no appearance of clot after addition of thrombin. Animals sacrificed shortly before the expected time of death, or having died after venom injection, had severe hemorrhages in the abdominal wall muscles, the peritoneum and the diaphragm; blood clots as well as free bloody fluid were found in the abdominal cavity. There were no gross or microscopic hemorrhages elsewhere in the body.

*Intravenous injection.* The $LD_{50}$ by the intravenous route was 11.5 μg per 20 g mouse, whereas 15 μg or more caused death of all the mice. In addition to the above described signs there was bleeding from the nose and mouth shortly before death. One $LD_{50}$ caused death after 4 to 24 hours, 3 $LD_{50}$ within 30 to 60 minutes. Animals sacrificed 10, 20, 30 and 60 minutes following the intravenous injection of 3 $LD_{50}$ had no anemia (hemoglobin 15–16 g %) but severe thrombocytopenia (35,000–6,000 per cmm) and their blood was incoagulable. Whereas after 10 minutes the lungs showed macroscopic punctate hemorrhages, with increasing intervals of time more extensive hemorrhagic areas were observed. When the animals were allowed to die, widespread hemorrhages were seen mainly in the lungs, to a slighter degree in the heart, the skin and the peritoneum, but not in the brain. Microscopic examination of the lungs revealed mainly perivascular hemorrhages forming cuffs around

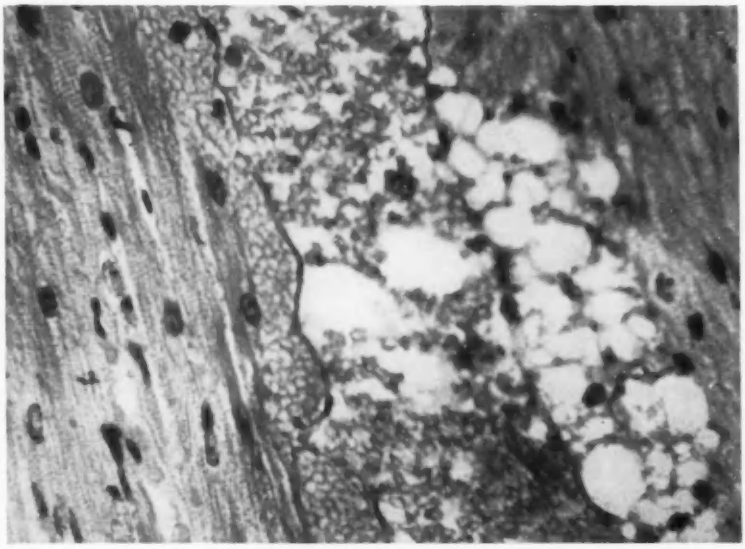

FIG. 1. Heart muscle of mouse (20 g) injected i.v. with 15 μg of venom. Death after 1.5 hours. Capillary with per diapedesim hemorrhage. H.E. 47.5 × 4.

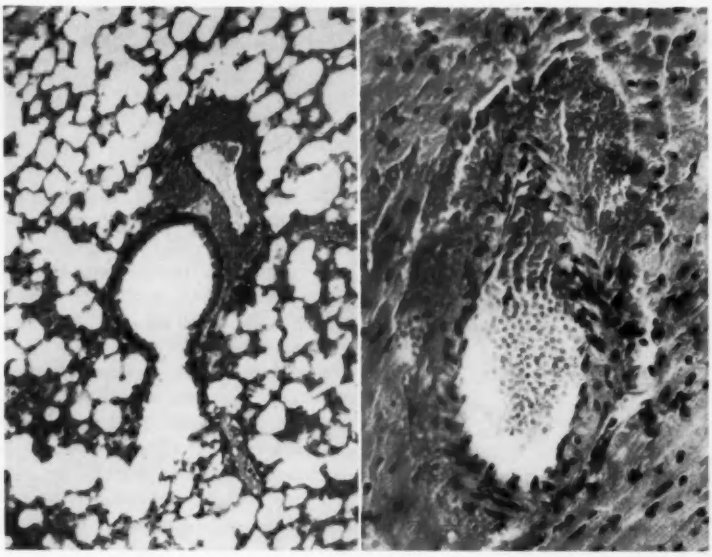

FIG. 2. Lung and heart muscle of mouse (20 g) injected i.v. with 15 μg of venom. Death after 2 hours. Cuffing of blood vessel by hemorrhage. Vessel wall almost intact. Left: lung H.E. 20 × 4. Right: heart H.E. 47.5 × 4.

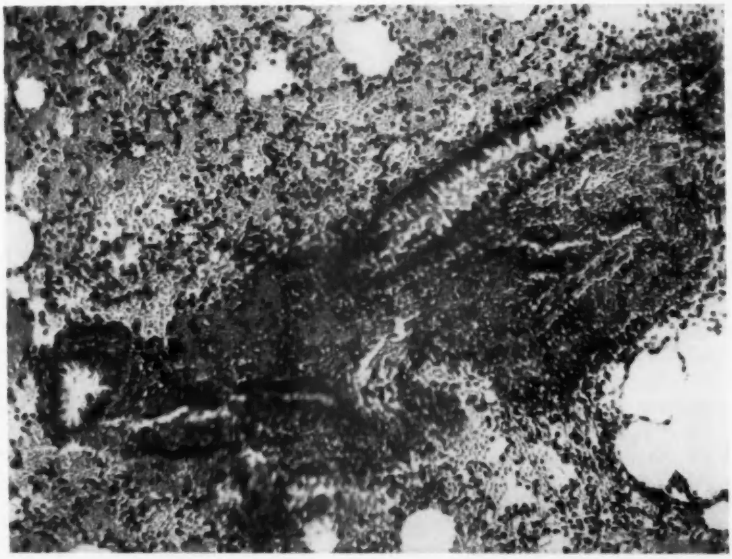

FIG. 3. Lung of mouse (20 g) injected i.v. with 15 μg of venom. Death after 2.5 hours. Marked damage of the vessel wall and perivascular hemorrhage. H.E. 20 × 4.

the bronchial vessels. In this "initial" stage of bleeding the vessel wall was morphologically still intact (Figures 1 and 2). Transitions from these cuff-like perivascular hemorrhages to massive bleeding were found. Stages of deterioriation of the vessel wall and immigration of leucocytes in the damaged vessel wall were apparent (Figure 3). On no occasion were intravascular blood clots noted.

### *In vitro activities*

*Various enzymes.* The following enzymatic activities were found: protease, L-amino acid oxidase and phosphatidase (minimal concentrations effective in the various systems 1 μg/ml, 20 μg/ml and 5 μg/ml respectively) and hyaluronidase (135 μg of venom equivalent to one turbidimetric unit). Addition of venom to washed red blood cells did not cause hemolysis. Fibrinolysin is referred to below under coagulation factors.

*Erythrocyte sphering factor.* Addition of venom to a red cell-plasma-polyvinylpyrrolidone system[10] caused red cell sphering, inhibition of rouleaux formation and of red cell sedimentation. The minimal concentration of *Echis* venom causing complete sphering and maximal inhibition of sedimentation was 200 μg/ml, while 20 μg/ml still caused red cell crenation and partial sedimentation inhibition. A red cell sphering action of the venom on whole blood could not be investigated as clotting interfered.

*Blood coagulation factors.* The venom has both procoagulant and anticoagulant activities and is both fibrinogenolytic and fibrinolytic.

Addition of venom to whole blood shortened the coagulation time at concentrations of 0.2 μg/ml up to 400 μg/ml, while from 1 mg/ml upwards, the blood became incoagulable, and did not clot on addition of thrombin. Venom clotted oxalated plasma also without addition of calcium ions and shortened the recalcification time at concentrations below 1500 μg/ml, while at 2500 μg/ml and higher concentrations, the plasma became incoagulable, and did not clot upon the addition of thrombin. Clot lysis was observed at concentrations of venom which clotted the oxalated plasma rapidly and accelerated the recalcification time markedly, the lysis time being more rapid at shorter clotting times (Figure 4).

Addition of venom to oxalated plasma in a final concentration of 2500 μg/ml caused the plasma to become incoagulable by thrombin within 30 seconds. Addition of venom in the same concentration to plasma clotted by recalcification or by thrombin caused complete lysis of the clot within 30 to 60 minutes. When, however, the venom was present at the time of formation of the clot, lysis at such and smaller concentrations was much more rapid (Figure 4). Venom was also able to lyse washed, heated plasminogen deficient fibrin clots, which were not lysed by streptokinase (10,000 units, 24 hours), demonstrating that the venom acts on the fibrin itself. Venom did not clot fibrinogen (Cohn fraction I) nor did it accelerate the conversion of fibrinogen to fibrin by thrombin. More detailed studies on the clot accelerating action of the venom carried out on the separated procoagulant, in order to obviate interference by the strong fibrinogenolysin, are given below.

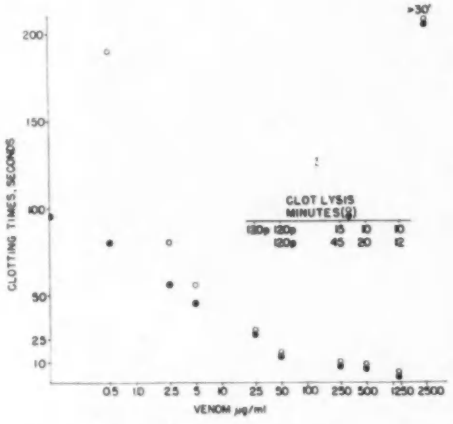


FIG. 4. Effect of venom on oxalated human plasma in the absence and presence of calcium ions. "○" in absence of calcium ions; "●" in presence of calcium ions. The numbers in the graph indicate time of complete lysis, with "p" partial lysis. Clotting system: 0.1 ml oxalated plasma + 0.05 ml venom solution + 0.05 $CaCl_2$ 0.05 M or saline.

### *Immunology*

*Specific antiserum.* The specific rabbit antiserum contained precipitating antibodies against whole venom in a dilution up to 1:512. With the gel diffusion technic 13 antigens were demon-

FIG. 5. Gel diffusion. *Echis colorata* venom against specific rabbit antiserum.

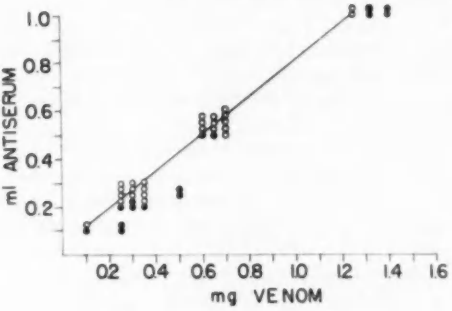


FIG. 6. Neutralization titration curve of anti-*Echis colorata* venom rabbit serum tested with whole venom.

strated (Figure 5). A similar result was obtained by immunoelectrophoresis.

Intraperitoneal neutralization tests with this antiserum in mice resulted in a straight neutralization line (Figure 6) following the law of multiple proportions,[18] indicating the presence of neutralizing antibodies against the main venom components responsible for the lethal action of the venom.

Mice thus protected did not show any of the clinical signs described above, had no hemorrhages in the peritoneum or elsewhere and their blood had a normal clotting time and a normal thrombocyte count. Mice injected intravenously with a venom (2 $LD_{100}$)-rabbit serum (0.2 ml) mixture did not become ill and did not show hemorrhages in the lung or elsewhere in the body; but, when examined 30 minutes after injection, their blood was found to be incoagulable and did not clot upon the addition of thrombin. They had thrombocytopenia (16,000/cmm) but no anemia.

The rabbit antiserum was capable of neutralizing the in vitro proteolytic activity (Gelatin test) of the venom, but not the amino acid oxidase, the phosphatidase, the erythrocyte sphering factor, the anticoagulant, the procoagulant and fibrinogenolysin-fibrinolysin. The effect of the antiserum on the hyaluronidase of the venom could not be examined as the serum albumin interfered with the test.

*Cross immunity with Vipera palestinae serum.* As no specific serum against *E. colorata* venom is available for therapy in the human, it seemed important to examine the Pasteur anti-*V. palestinae* horse serum[17] for cross reaction with a view to its potential value in cases of bite by *E. colorata*.

In the gel diffusion the Pasteur anti-*V. palestinae* horse serum gave 4 to 5 lines with the *E. colorata* venom (this Pasteur serum is monovalent, specific for *V. palestinae*). An even higher number of common precipitation lines was found in immunoelectrophoresis. The anti-*E. colorata* rabbit serum produced in gel diffusion only 2 lines against *V. palestinae* venom.

The anti-*V. palestinae* serum was able to neutralize in vitro the *E. colorata* protease. Its in vitro neutralizing potency, however, was at least 10 times weaker than that of homologous serum.

In the in vivo neutralization test the anti-*V. palestinae* serum was not able to prevent death by *E. colorata venom*. A definite effect, however, was observed, in that the survival time of the mice was prolonged from less than one-half hour (6 $LD_{50}$ of *E. colorata* venom intraperitoneally) to 24 hours (6 $LD_{50}$ *E. colorata* venom + 0.25 ml anti-*V. palestinae* serum). The peritoneal hemorrhages in these animals were relatively slight.

### *Chromatographic fractions*

Details of the chromatography on DEAE-cellulose are presented in a previous study on *V. palestinae* venom.[13] After equilibration with a starting buffer of pH 8.2, 0.005 M, 4.25 ml of dialyzed venom corresponding to 2.5 ml 10% stock solution was applied to the column (17 x 1.4 cm) with a maximum velocity of 10 ml per hour, and followed by 60 ml starting buffer with the same velocity. Subsequently, 60 ml of phosphate buffer 0.005 M, pH 6.8 was passed after which gradient elution was started at the same velocity with 600 ml of phosphate buffer, pH 6.8 (300 ml of 0.005 M and 300 ml 0.2 M) as indicated in "methods". When 225 ml had been collected, the

gradient elution was interrupted (in preliminary runs this was sufficient to elute the two main fractions, see below) and 50 ml 1 M NaCl was passed. The eluate was collected in 5 ml samples.

The chromatographic pattern of the fractions obtained from 250 mg *E. colorata* venom is illustrated in Figure 7.

Two fractions were obtained, the first containing lethal and hemorrhagic activity, anticoagulant, protease and the bulk of the amino acid oxidase and of the fibrinolysin. The second fraction contained the procoagulant, most of the lethal, hemorrhagic and protease activities and a weak fibrinolytic and amino acid oxidase activity. The first fraction contained two pigments, a yellow and a violet one. The second fraction had a pale yellow color. Hyaluronidase was not recovered; it was found, however, to have been lost already during the dialysis of the venom preceding chromatography. The phosphatidase and the red cell sphering factor, which had been present in the dialysate, were lost during chromatography.

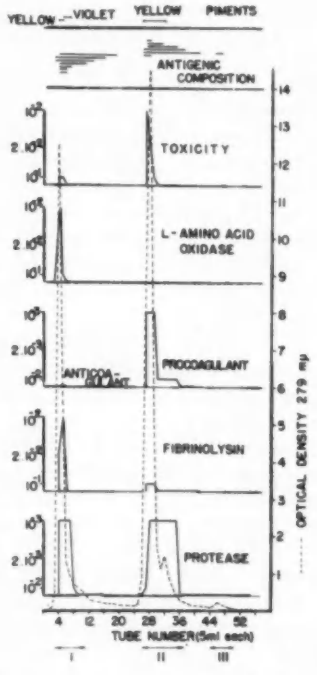


FIG. 7. Chromatogram of 250 mg of *Echis colorata* venom on DEAE-cellulose.

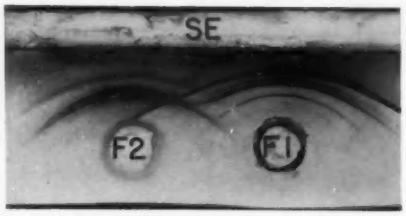


FIG. 8. Gel diffusion. Chromatographic *Echis colorata* venom fractions against anti-*Echis colorata* rabbit antiserum.

The calculated recovery of lethal activity was 63% ($LD_{100}$ applied 7500, recovered 4775, of which 575 was in the first fraction and 4200 in the second).

There was a marked difference in the toxic effects of the two fractions. Fraction 1 caused severe peritoneal hemorrhages with a large amount of free fluid in the abdominal cavity. Fraction 2, on the other hand, produced only slight hemorrhages in the peritoneum with only a small amount of free bloody fluid but with a large ramified blood clot in the abdomen.

*Blood coagulation factors.* The properties of the anticoagulant were similar to that of the whole venom and its action could be explained by fibrinogenolytic action. The procoagulant fraction 2, like whole venom, clots oxalated plasma but does not clot fibrinogen and does not accelerate the conversion of fibrinogen to fibrin by thrombin. It clots oxalated hemophilic A plasma as rapidly as normal oxalated plasma, but it is ineffective on oxalated plasma treated with $BaSO_4$. It is probable, therefore, that the procoagulant acts by converting prothrombin to thrombin. In stored plasma the procoagulant is much less effective, clotting being markedly delayed. Whereas 0.1 ml fresh oxalated plasma was clotted by 0.05 ml procoagulant fraction in less than 5 seconds, plasma stored at 4°C for 30 days (one-stage prothrombin time 50 seconds, two stage prothrombin content 75%) was clotted by the procoagulant in 49 seconds. Apparently factor V is required for the formation of thrombin from prothrombin by the venom.

It is noteworthy that the procoagulant fraction 2 is also fibrinogenolytic and fibrinolytic, although much weaker than the anticoagulant fraction. Indeed, at no procoagulant concentration, was prolongation of clotting time due to fibrinogenolysis observed.

*Immunology.* Gel diffusion against anti-*E. colorata* rabbit serum showed that, after chromatography, 12 different antigens were recovered, as compared with the 13 antigens in the whole venom. The first fraction contained 7 antigens and the second 6, one antigen being identical for both fractions (Figure 8).

In vivo neutralization tests showed that for the neutralization of 1 $LD_{100}$ of fraction 1, the amount of anti-*E. colorata* rabbit serum required was about 3 times larger than that required for whole venom and for fraction 2. As for whole venom, only the protease of both fractions, and not the procoagulant, the anticoagulant or the fibrinogenolytic and fibrinolytic activities, was neutralized by the homologous serum in vitro.

In vivo neutralization tests with anti-*V. palestinae* horse serum against *E. colorata* venom fractions showed that the serum did not protect against the lethal action of fraction 1 (3.5 $LD_{100}$, 0.5 ml serum), whereas it did protect against that of fraction 2 (6 $LD_{100}$, 0.25 ml serum effective). Gel diffusion of separate *Echis* venom fractions against heterologous serum showed 2 precipitation lines with fraction 1 and 3 with fraction 2.

## DISCUSSION

Although severe hemorrhages were invariably found in mice which had died following the intraperitoneal or intravenous injection of the *E. colorata* venom or fractions, it does not follow necessarily that the lethal action of the venom is due to its hemorrhagin only. In 1935 it was shown by Taylor and Mallick[3] that the hemorrhagins of *Echis carinata* and *Vipera russellii* venoms are not the principal lethal agents as heterologous antisera, capable of neutralizing the hemorrhagins, did not prevent rapid death. An important role was ascribed by these authors and by Chopra *et al.*[6] to other lethal agents, presumably neurotoxins. It is possible, although not yet established, that also the signs of nervous system disturbance, such as restlessness, paresis and uncoordinated movements observed after injection of *E. colorata* venom, are due to a neurotoxin. No neurotoxin, however, could be separated by chromatographic fractionation of the venom.

In the explanation of the hemorrhages produced by the venom, the afibrinogenemia, the thrombocytopenia and a hemorrhagin (cytotoxin) damaging the vessel wall and possibly related to the protease[19] must be considered.

Chopra *et al.*[6] considered fibrinolysis important in the causation of blood incoagulability of cats injected intravenously with *E. carinata* venom. Taylor *et al.*,[4] on the other hand, ascribed the afibrinogenemia occurring following injection of *E. carinata* venom in pigeons and monkeys to intravascular clotting due to a procoagulant. Whereas these latter authors occasionally observed intravascular clots when using large doses of *E. carinata* venom, in this study intravascular clots were never seen in mice dying with afibrinogenemia following injection of *E. colorata* venom. Although this may have been due to rapid intravascular clot lysis, the observation that injection of fraction 1, which is strongly fibrinogenolytic but not procoagulant, did cause afibrinogenemia points to the possibility that the afibrinogenemia appearing after injection of whole venom is mainly a result of the whole venom's fibrinogenolytic action. The differences among the in vivo actions of blood coagulation factors of various Viperidae venoms may well be due to the relative strengths of the venoms' procoagulant and fibrinolysin.

There are strong arguments favoring a hemorrhagin as the principal or primary hemorrhagic factor. First, although following intraperitoneal injection of the venom, incoagulability and thrombocytopenia of the systemic blood do appear, the hemorrhages in the abdominal muscles, the peritoneum and the diaphragm are the most striking pathological changes. Secondly, the morphological evidence of per diapedesim hemorrhage in the lungs and in the heart muscle following intravenous injection of the venom points to a factor first affecting the intercellular spaces of the vascular endothelium, and subsequently damaging the whole vessel wall. Thirdly, following intravenous injection of a mixture of venom and specific rabbit antiserum, no hemorrhages appeared and the animals survived without developing anemia, although the blood became thrombocytopenic and afibrinogenemic (the serum has no antibody against the fibrinolysin). The afibrinogenemia and thrombocytopenia may be considered, therefore, contributing factors in the pathogenesis of the hemorrhages.

In this context it is of interest to consider a possible interdependence between the hemorrhagin on the one hand and the fibrinogenolysin and thrombocytopenia producing factor on the other. Although the rabbit serum could not neutralize the fibrinogenolysin nor the thrombocytopenic

factor of the venom as shown in the intravenous experiment, intraperitoneal injection of a venom-serum mixture did not lead to afibrinogenemia or to thrombocytopenia. One explanation may be that the fibrinogenolysin and the thrombocytopenic factor can penetrate through the blood vessel walls only when the walls have been damaged by the hemorrhagin.

Chromatographic fractionation of the venom showed that both the phosphatidase and the amino acid oxidase (the latter in agreement with the observation of Singer and Kearney[20]) are not essential for the lethal action of the venom.

The coagulation factors of the venom of *E. colorata* apparently resemble closely those of *E. carinata venom*,[6, 7] which is also capable of both clotting decalcified plasma and lysing fibrin. These venoms differ from *V. palestinae* venom[13] which accelerates the conversion of fibrinogen to fibrin in the presence of thrombin and inhibits thrombin formation.[11] The procoagulant action of the *E. colorata* venom resembles closely that of *V. russellii*, in that it converts prothrombin to thrombin, but is not able to normalize the clotting time of stored plasma.[21, 22] The anticoagulant action of Russel viper venom, however, is ascribed by Wenckert and Nilsson[22] to the formation of an anticoagulant from prothrombin, while in the case of *E. colorata* fibrinogenolysis is the most probable explanation.

The straight line neutralization obeying the law of multiple proportions,[18] obtained with the specific rabbit serum for *E. colorata* venom doses up to 36 $LD_{100}$, is promising from the point of view of therapy, as it indicates adequate antigenicity of all lethal factors. Until a commercial specific serum becomes readily available, however, anti-*V. palestinae* serum,[17] shown to possess antibodies against some *E. colorata* venom components and capable of delaying death in mice, may be used. Similar cross-immunity among the venoms of other Viperidae has been demonstrated by Taylor and associates,[3, 5] and by Izard and Boquet.[8]

SUMMARY

Toxicological, biochemical and immunological studies were carried out on the venom of *Echis colorata* Günther 1878 and its chromatographic fractions.

The in vivo effects of the venom in mice were hemorrhage, nervous system disturbance, anemia, thrombocytopenia and afibrinogenemia. The $LD_{50}$ in mice weighing 20 g was 31 μg by the intraperitoneal route and 11.5 μg by the intravenous route. In vivo activities of the venom were protease, phosphatidase, erythrocyte sphering, hyaluronidase, L-amino acid oxidase, procoagulant, anticoagulant, fibrinogenolysin and fibrinolysin.

Evidence is presented that the hemorrhages are mainly due to damage of the blood vessel walls, whereas the afibrinogenemia and the thrombocytopenia are contributing factors.

A specific rabbit antiserum was able to neutralize the lethal activity, the hemorrhagic activity and the protease of the venom, but not the other forementioned activities. Partial cross immunity with anti-*Vipera palestinae* horse serum was established.

ACKNOWLEDGMENT

We are indebted to Dr. H. Mendelssohn and to Dr. A. Tur-Kochba, the Institute of Natural Sciences, Department of Biology, University of Tel-Aviv, for providing the venom of *Echis colorata*.

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# GENERALIZED HISTOPLASMOSIS IN A PANAMANIAN INFANT: CASE REPORT

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Histoplasmosis was unknown prior to Darling's description from Panama in 1906.[1, 2] His three reported cases found in adults at autopsy were the only instances of recognized histoplasmosis from this area until 1951 when Draheim[3] reported a fourth in a 4 month old Panamanian infant. One further case of disseminated histoplasmosis in a 29 year old Navy enlisted man, stationed in the Canal Zone, was reported in 1956 by Peabody.[4] Young in 1957[5] referred to four additional fatalities in adults, confirmed by autopsy at Gorgas Hospital. Of the above patients, only the child was a native born Panamanian.

This report deals with some of the unusual aspects found in the second known case of fatal histoplasmosis to occur in a Panamanian infant.

A. O., a 7 month old male of Indian parentage, was admitted to Amador Guerrero Hospital, Colon, R. de P., on 13 April 1959. The parents reported that the infant had had fever and cough for 2 weeks.

Initial physical examination was significant only in the finding of pallor and hepato-splenomegaly (1 to 2 fingerbreaths below the costal margins). The chest was clear. Daily temperature elevations to 40°C were noted throughout the period in hospital. No other remarkable findings were recorded.

The patient was first seen in consultation by one of the authors on 30 April 1959, 17 days after admission. He appeared acutely ill, with marked pallor, and was noted to roll his head from side to side. The neck was resistant to flexion. Hepato-splenomegaly was present; both organs were firm and felt about 2 cm below the costal margins. The remainder of the examination was negative.

Laboratory studies performed at Amador Guerrero Hospital at the time of admission included hemoglobin of 6 g %; leukocytes 5,300 with 90% polymorphonuclear cells, 10% lymphocytes; stool negative for ova and parasites; urinalysis negative and typhoid agglutination test negative. One week later a repeat typhoid agglutination test was again negative and a leukocyte count was 5,300 with 38% polymorphonuclear cells, 56% lymphocytes, 4% myelocytes, 2% eosinophiles. Brucella and typhus agglutination tests and malaria smears were negative. Eleven days after admission cerebrospinal fluid contained 31 lymphocytes per cmm. X-ray films of the chest on 20 April and 24 April were unsatisfactory, but the film on 4 May was not remarkable. A tuberculin skin test (6 May) was negative.

At the time of consultation (30 April), even though the histoplasmin skin test was negative (29 April), blood and iliac bone marrow specimens were obtained for diagnostic studies. The bone marrow specimen was cultured directly on Mycosel blood agar and brain heart infusion dextrose broth at 25° and 37°C. In addition, 0.2 ml was inoculated intraperitoneally into each of two mice. One mouse was sacrificed 2 weeks and the other 3 weeks after inoculation. Liver and spleen were removed, homogenized separately, and cultured on Mycosel blood agar. The patient's blood was cultured in brain heart infusion dextrose broth at 37°C with subculture to Mycosel blood agar and Sabouraud's dextrose agar 2 weeks later.

Characteristic colonies of *Histoplasma capsulatum* were observed on the Mycosel agar 9 days after seeding with bone marrow and microscopic examination of the fungus confirmed the identification. Within a period of 1 month *H. capsulatum* was recovered from every tube of medium inoculated with the patient's bone marrow and blood, as well as with liver and spleen tissue from both mice.

Complement-fixation tests for histoplasmosis were performed on the serum collected 30 April, using the 50% endpoint test and employing two antigens. A serum titer of 1:32 was obtained with the whole yeast phase antigen and one of 1:16 with the histoplasmin antigen.

The child had received penicillin and streptomycin for 10 days starting on 13 April. This was followed by 6 days of oxytetracycline (300 mg per day). Whole blood (200 ml) was given on

two occasions (20 April and 4 May). He continued to have daily temperature elevations to 40°C and his condition remained poor. On 9 May he was signed out of the hospital by his parents and returned to his family home in a primitive native village about 25 miles west of Colon, R. de P., in an area impossible to reach by normal transportation. On that day *H. capsulatum* was identified in the cultures of blood and bone marrow. The family and child were located but re-hospitalization of the infant was refused. Approximately 3 weeks after his return home the infant expired. No autopsy was obtained.

In view of the isolation of *H. capsulatum* from bone marrow and blood the diagnosis of generalized histoplasmosis was made, supported by the anemia, leukopenia, hepato-splenomegaly, and the clinical course. This, the second case reported in a native Panamanian infant, could easily have remained unrecognized on the basis of the negative histoplasmin skin test and x-rays of the chest. The anergy frequently found in terminal histoplasmosis is well known, resulting in lack of histoplasmin sensitivity. The lack of positive findings in x-rays of the chest has also been reported.[6]

Histoplasmosis is known to be endemic on the Isthmus of Panama.[4, 5, 7, 8] Current skin test surveys (unpublished), in the Canal Zone Latin American schools, have shown up to 60% histoplasmin hypersensitivity in the Panamanian children attending the first grade. These data indicate an early exposure to the fungus, and the present case illustrates the existence of the disease in this age group. Undoubtedly there are many cases of unrecognized active histoplasmosis in pre-school children and infants.

SUMMARY

A fatal case of generalized histoplasmosis in a 7 month old Panamanian infant is presented. In spite of negative histoplasmin skin test and x-ray of the chest, cultural studies were undertaken resulting in isolation of *H. capsulatum* from the patient's bone marrow and blood. Compatible diagnostic titers were obtained in the complement-fixation test.

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# THE "LUNG SHIFT" OF *SCHISTOSOMA MANSONI* IN MICE FOLLOWING THERAPY WITH TARTAR EMETIC OR MIRACIL D

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During studies on the effects of various chemical compounds on *Schistosoma mansoni* infections in mice, it was noted frequently that the total worm burden in treated animals was less than that of untreated controls, often within 24 to 48 hours after the initial dosage. The widely accepted procedure of enumerating worms found in the mesentery, portal vein, and liver at necropsy was employed.[1-7] It seemed unlikely that the "missing worms" in the treated mice could be accounted for solely by their rapid death and dissolution as a result of therapy, particularly since many male and female worms in the liver, although opaque and motionless, were still intact at early periods of necropsy. It was recognized that wide individual variability in total worm counts occurred among mice inoculated with comparable numbers of cercariae, but the chances were slight of consistently selecting at random lighter infections for the treated groups.

In attempts to account for such observed differences, one of us (E. G.) initiated examinations of visceral sites other than the mesentery, portal vein, and liver. As a result, mature males and females, both living and dead, were found within the lungs, hearts, spleens, and kidneys of mice at various periods after treatment, with notably greater numbers in the lungs. Some worms also occurred in these sites in untreated controls, but in much smaller proportions relative to the total worm burden. It became apparent that a lung shift of *S. mansoni* in mice following therapy, with at least some types of chemical compounds, and under the conditions of our experiments, was equally as consistent as the hepatic shift described by other investigators.

Pulmonary involvement has been observed previously during the course of infections with *S. mansoni* in both laboratory animals and man,[3, 8-13] aside from the migratory movement of immature stages through the lungs during early periods of post-cercarial development. To our knowledge, the passage of large numbers of adult schistosomes into the lungs as a direct consequence of chemotherapy has not been emphasized.

The present paper concerns the lung shift of *S. mansoni* in mice at various intervals following therapy with tartar emetic and 1-diethylamino-ethylamino-4-methylthioxanthone (Miracil D).

## METHODS

Female white mice (Manor-Swiss strain), weighing from 18 to 22 g, were inoculated intraperitoneally with 80 to 90 cercariae of *S. mansoni* obtained from experimentally infected snails (*Australorbis glabratus*). At periods after inoculation varying from 8 to 12 weeks, the mice were segregated into appropriate groups and were treated for varying periods of time as indicated in the accompanying tables. The doses of tartar emetic and Miracil D employed were known from previous studies to be highly effective against *S. mansoni*.

After intervals of from 1 day to 4 weeks following the first dosage, representative proportions of the surviving mice, in groups of 5 or 10, were killed and necropsied. The living and dead worms in the mesentery, portal vein, liver, and lungs were counted, with notations on sex distribution and the occurrence of worms singly or in pairs. In some cases, the heart, spleen, and kidneys were also examined for the presence of worms (since relatively few worms occurred in these alternative sites, and did not account for large proportions of the total worm burdens, the data are not included in the tables).

In the search for worms in the lungs and liver, the tissue was torn apart carefully with fine dissecting tweezers, or the entire organ was pressed and examined between two 5″ x 5″ glass plates. Completely immobile and opaque worms, with or without overt signs of disintegration, were listed as dead.

For purposes of simplification, the mean values for the various categories listed in the tables are

* With the technical assistance of Elizabeth Schumacher.

expressed as the nearest whole numbers, and the standard errors of the means are given to the first decimal place only. Statistical comparisons for the significance of differences between groups of five or ten treated and untreated mice were performed by the ranking test described by Wilcoxon.[14]

## RESULTS

*Distribution of mature* S. mansoni *in untreated mice.* The location of mature schistosomes in 85 untreated mice (17 groups of five mice each), at intervals of from 39 to 112 days after inoculation with 80 to 90 cercariae, followed a fairly uniform pattern over the period of several months included in this study. As demonstrated by the data for untreated mice in Tables 1 and 2, considerable variation occurred in the number of worms found in individual animals, but the vast majority of these worms occurred in the mesentery and portal vein; negligible proportions of the total worm burdens occurred in the liver. The tables illustrate some variation in the percentage of worms present in the lungs, but rarely did this exceed 10% of the total burden, and a mean of 1 to 4% among five or ten mice was common. The over-all mean percentage of worms in the lungs of 85 untreated controls was 2 ± 1.4 (standard error).

*Distribution of mature* S. mansoni *in treated mice.* Within 24 hours after the initiation of therapy with twice-daily intraperitoneal doses of 20 mg/kg of tartar emetic, a marked shift of schistosomes from the mesenteric veins occurred (Table 1). At the first necropsy period (1 day after the first dose), approximately equal proportions of the worm burden were distributed between the portal vein, liver, and lungs, and no worms whatever were recovered from the mesentery. On the 3rd and 15th days after the first dose, the hepatic shift was more marked, but the percentage of worms within the lungs remained somewhat similar to that observed on the 1st day. Table 3 illustrates the variability in the degree of the hepatic and lung shifts among ten treated mice. Also illustrated in Table 3 is the predominance of unpaired worms, a significant feature of antischistosome activity which usually occurs shortly after therapy is started.

At the 40 mg/kg level of tartar emetic, administered intraperitoneally, twice daily for 1 day, and once on the following day (a total of three doses), a pronounced shift of worms to the lungs was observed 4 weeks after the first

TABLE 1

*Hepatic and lung shifts of* Schistosoma mansoni *in mice following treatment with tartar emetic*

| Mg/kg Ip. B.I.D. × no. doses | Necropsy day after first dose | Means§ ± standard errors | | | | |
|---|---|---|---|---|---|---|
| | | Total no. of worms | % distribution of worms | | | |
| | | | Mesentery | Portal vein | Liver | Lungs |
| 20 × 3 | 1 | 24 ± 2.5* | 0 | 29 ± 4.6 | 35 ± 7.5 | 36 ± 8.2 |
| 20 × 7 | 3 | 36 ± 4.4 | 0 | 8 ± 2.4 | 69 ± 6.7 | 23 ± 6.1 |
| 20 × 10 | 15 | 31 ± 5.4 | 0 | 3 ± 2.7 | 65 ± 10.9 | 32 ± 15.6 |
| 40 × 3‡ | 31 | 26 ± 1.3† | 0 | 12 ± 8.1 | 39 ± 14.8 | 49 ± 14.9 |
| — | 1 | 36 ± 2.6 | 28 ± 3.4 | 66 ± 4.4 | 4 ± 4.4 | 2 ± 1.7 |
| — | 3 | 33 ± 2.3 | 33 ± 2.4 | 65 ± 3.3 | <1 | 2 ± 1.5 |
| — | 15 | 29 ± 1.2 | 34 ± 3.1 | 63 ± 3.8 | 2 ± 0.8 | 1 ± 0.9 |
| —‡ | 30 | 35 ± 2.3 | 55 ± 3.0 | 43 ± 4.7 | 0 | 2 ± 2.3 |
| Reference standards‖ 85 untreated mice | | 36 ± 2.1 | 35 ± 2.5 | 63 ± 2.5 | <1 | 2 ± 1.4 |

* p = 0.01.

† p = 0.05; statistically significant differences from the untreated controls.

‡ 5 mice per group; all others, 10 mice per group.

§ Expressed as the nearest whole numbers.

‖ From 17 groups of 5 mice each, 39–112 days after intraperitoneal inoculation with 80–90 cercariae.

Ip. = intraperitoneally, B.I.D. = twice daily, < = less than.

TABLE 2

*Hepatic and lung shifts of* Schistosoma mansoni *in mice following treatment with Miracil D*

| Mg/kg ×† no. doses | Necropsy day after first dose | Means‡ ± standard errors | | | | |
|---|---|---|---|---|---|---|
| | | Total no. of worms | % distribution of worms | | | |
| | | | Mesentery | Portal vein | Liver | Lungs |
| 300 Or. × 1 | 1 | 41 ± 7.5 | 26 ± 9.0 | 55 ± 8.1 | 9 ± 2.8 | 10 ± 3.3 |
| 300 Or. × 1 | 10 | 36 ± 4.5 | 0 | 20 ± 8.0 | 61 ± 5.1 | 19 ± 6.8 |
| 300 Or. × 1 | 17 | 43 ± 5.6* | 0 | 6 ± 2.7 | 42 ± 10.6 | 52 ± 9.3 |
| 300 Or. × 1 | 24 | 26 ± 4.2 | 0 | 10 ± 2.3 | 45 ± 8.4 | 45 ± 9.9 |
| 40 Ip. × 10‖ | 30 | 28 ± 7.5 | 0 | 0 | 42 ± 7.3 | 58 ± 7.3 |
| — | 1 | 38 ± 5.9 | 45 ± 5.0 | 50 ± 4.9 | 0 | 5 ± 1.3 |
| — | 10 | 37 ± 7.4 | 50 ± 7.1 | 49 ± 7.5 | 0 | 1 ± 0.3 |
| — | 17 | 28 ± 3.0 | 46 ± 6.7 | 47 ± 3.6 | 0 | 7 ± 4.0 |
| — | 24 | 22 ± 3.7 | 38 ± 4.1 | 58 ± 4.4 | 0 | 4 ± 2.5 |
| — | 30 | 35 ± 2.3 | 55 ± 3.0 | 43 ± 4.7 | 0 | 2 ± 1.4 |
| Reference standards§ 85 untreated mice | | 36 ± 2.1 | 35 ± 2.5 | 63 ± 2.5 | <1 | 2 ± 1.4 |

* $p = 0.05$, a statistically significant difference from the untreated controls.
† 5 mice per group.
‡ Expressed as the nearest whole numbers.
§ As in Table 1.
‖ Twice daily for 5 days.
Or. = orally, Ip. = intraperitoneally, < = less than.

TABLE 3

*Variability of distribution of* Schistosoma mansoni *in the visceral organs of ten treated mice*

| Mouse no. | Total no. of worms | % distribution of worms† | | | | % unpaired worms |
|---|---|---|---|---|---|---|
| | | Mesentery | Portal vein | Liver | Lungs | |
| 1 | 20 | 0 | 25 | 15 | 60 | 80 |
| 2 | 35 | 0 | 28 | 11 | 61 | 31 |
| 3 | 22 | 0 | 23 | 18 | 59 | 73 |
| 4 | 27 | 0 | 30 | 70 | 0 | 63 |
| 5 | 15 | 0 | 20 | 74 | 6 | 86 |
| 6 | 17 | 0 | 53 | 35 | 12 | 70 |
| 7 | 19 | 0 | 15 | 38 | 47 | 90 |
| 8 | 21 | 0 | 24 | 48 | 28 | 81 |
| 9 | 37 | 0 | 13 | 26 | 61 | 70 |
| 10 | 27 | 0 | 56 | 18 | 26 | 60 |
| Means* ± standard errors | 24 ± 2.4 | 0 | 29 ± 4.6 | 35 ± 7.5 | 36 ± 8.6 | 70 ± 5.3 |

* Expressed as the nearest whole numbers.
† 24 hours after the first of 3 intraperitoneal doses of 20 mg/kg of tartar emetic.

dosage (Table 1). About 50% of the total worm burden occurred in the lungs of five treated mice, as compared with 2% in the lungs of five corresponding untreated controls. Examinations at earlier necropsy periods were not performed following this dose level.

After single oral doses of 300 mg/kg of Miracil D, large numbers of worms were not recovered from the lungs until the 17th-day necropsy period (Table 2). Although the worms apparently moved from the mesenteric veins sometime between the 1st and 10th days, with a marked hepatic shift at the latter period, the rate of transfer to the lungs was slower than that following multiple intraperitoneal doses of tartar emetic. This difference may merely reflect the different dosage regimens employed with the two compounds. On the 24th day after the one-dose oral treatment with Miracil D, a large proportion of the worms were found within the lungs. Four weeks after the initiation of multiple intraperitoneal treatments (40 mg/kg twice daily for 5 days) with this compound, all of the worms were in the liver or lungs, with an approximately equal distribution between these two organs (Table 2).

Throughout Tables 1 and 2 it will be noted that the distribution of worms within the various organs in the untreated controls remained fairly constant at various periods of necropsy, following in general the trend of the reference control standards. The numbers of worms recovered from the controls, as well as from the treated mice, were somewhat more variable. In general, and allowing for the variability within groups of five or ten animals, the inclusion of the lung shift brought the total worm burdens in treated animals to levels nearly equivalent to or surpassing those of the untreated controls. A statistically significant difference between the worm burdens of

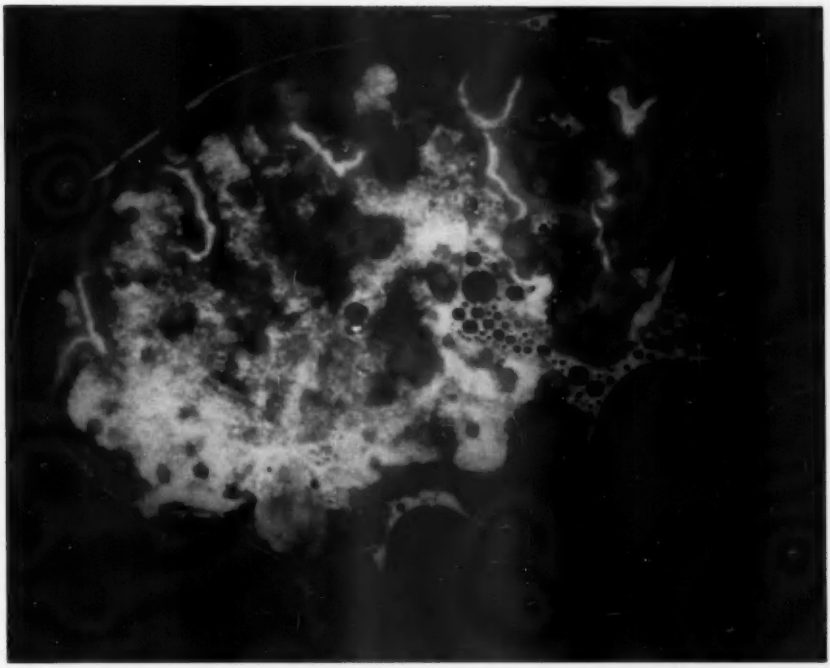

FIG. 1. A lung press preparation containing at least seven mature worms, from a schistosome-infected mouse 3 weeks after a single oral dose of 300 mg/kg of Miracil D. Only one lobe of the lungs is illustrated; seventeen dead and dying worms were found in the entire organ (31% of a total burden of 54 worms throughout the animal). Magnification × 5. Lightly tinged with Biebrich Scarlet and photographed through a green filter.

TABLE 4

*Sex distribution and mortality of* Schistosoma mansoni *in treated mice, within the liver and lungs*

| Treatment | Mg/kg × no. of doses | Necropsy day after first dose | No. mice per group | Total no. of worms† (% dead worms) | | | |
|---|---|---|---|---|---|---|---|
| | | | | Liver | | Lungs | |
| | | | | Males | Females | Males | Females |
| Tartar emetic | 20 Ip. × 7* | 3 | 10 | 162 (5) | 82 (0) | 61 (11) | 27 (15) |
| | 20 Ip. × 10* | 15 | 10 | 136 (68) | 57 (72) | 82 (82) | 46 (74) |
| | 40 Ip. × 3* | 31 | 5 | 39 (95) | 11 (91) | 40 (80) | 21 (100) |
| Miracil D | 100 Or. × 1 | 10 | 5 | 69 (74) | 41 (53) | 13 (31) | 17 (23) |
| | 100 Or. × 1 | 17 | 5 | 56 (83) | 29 (62) | 56 (95) | 62 (90) |
| | 100 Or. × 1 | 24 | 5 | 45 (93) | 10 (70) | 32 (91) | 30 (77) |
| | 40 Ip. × 10* | 30 | 5 | 41 (90) | 12 (92) | 45 (100) | 43 (86) |

* Twice daily.

† 80–90% were unpaired.

Ip. = intraperitoneally, Or. = orally.

ten treated and ten control mice occurred at the 1st-day necropsy period following the 20 mg/kg level of tartar emetic (Table 1). At this early period during therapy it is not suspected that the apparent reduction of worms in the treated group reflected the activity of the compound, and it is more likely that some of the worms were overlooked or that the treated group had fewer worms than did the controls at the onset of treatment. A second statistically significant reduction in total worm counts, as shown in Table 1, was observed at the 4-week necropsy period following administration of 40 mg/kg doses of tartar emetic. In this case, it is possible that some of the worms in the treated group had died and disintegrated, and were no longer recognizable.

At three different necropsy periods after Miracil D-treatment, more worms were recovered from treated animals than from the corresponding untreated controls (Table 2). In one of these cases (17th day), this difference was statistically significant. Wide deviations in the degrees of infections within small groups of treated and untreated mice account for such seeming anomalies.

The apparent slight lung shift on the 1st day after Miracil D-treatment was not a statistically significant difference from that of the corresponding untreated controls. All of the remaining lung shifts and the hepatic shifts, illustrated in Tables 1 and 2, were highly significant.

*Disposition of* S. mansoni *within the lungs.* No studies have been made as yet regarding the precise locations of the worms found in the pulmonary vascular system during and after therapy (*i.e.*, veins, arteries, or possibly extravascular locations). In most cases, little or no gross damage was observed in the areas adjacent to living worms, whereas hemorrhage and evidences of pneumonic consolidation of the lung parenchyma occurred in the vicinity of some of the dead worms.

Data are given in Table 4 on sex distribution and the percentage of dead worms in the lungs and livers of mice after treatment with tartar emetic and Miracil D. The majority of worms in these sites were unpaired. Only a few worms occurred in the lungs and livers of control mice at corresponding periods of necropsy, and all those present were living.

In the tartar emetic-treated mice, males outnumbered females in both the liver and lungs. After Miracil D treatment, male worms were more numerous than females in the liver, particularly at the last two necropsy periods, but the proportion of the two sexes in the lungs was similar throughout. In both sets of experiments, the numbers of males and females found in the mesenteries and portal veins of control mice were approximately equal. The predominance of males in the livers of both treated groups and in the lungs of the tartar emetic-treated animals may have been due in part to the more rapid death and dissolution of the females. On the other hand, no such inference can be drawn regarding the female worms in the lungs of the Miracil D

treated mice, since the numbers of males and females in this site, living and dead, were comparable.

## DISCUSSION

The pronounced lung shifts described herein have proved useful in evaluating antischistosome activity in mice at various periods following chemotherapy, supplementing the usual examination at necropsy of the mesentery, portal vein, and liver. The inclusion of worm counts from the lungs not only decreases the sometimes wide divergence between total worm burdens in treated animals and controls when only the mesenteric and portal vascular systems are examined, but additional dead or dying worms found within the lungs may enhance estimates of the degree of antischistosome activity. Such lung shifts appear to be a common response to treatment, and are not restricted to tartar emetic and Miracil D. Complete dosage-effect data have not been obtained with any one type of antischistosome compound, but preliminary information indicates that marginally-effective doses of some substances elicit only slight shifts of worms to the lungs, even though the hepatic shift may be prominent.

Among several questions which arise in interpreting the lung shift following therapy are those which concern the route, or routes, which allow some of the worms to progress beyond the liver whereas others die and are phagocytized in hepatic tissue.

In a recent publication by Standen[7] it is postulated that the worms in the mesenteric veins of mice lose considerable muscle tone as a result of therapy, and are unable to retain their hold on the walls of the blood vessels. Consequently, they are swept back into the liver. The precise reasons for the loss of sucker function, as well as for other physiological abnormalities caused by treatment, are not known.

Once within the liver, the worms may succumb to the effects of the antischistosome compound employed, or may recover eventually and return to the mesenteric veins. A third alternative is the passing of worms through or around the liver and their eventual lodgment within the pulmonary blood vessels.

Speaking of occasional worms (*S. mansoni*) which they found in the lungs of untreated mice, Moore *et al.*[3] considered that the young worms were either trapped there during early stages of migration and continued to develop, or that adult worms migrated from the portal to the systemic circulation. Similarly, in rabbits and hamsters, Yolles *et al.*[13] thought the route to the lungs might be through anastomoses from the portal to the systemic circulation. Referring to *S. haematobium* in monkeys, Fairley[10] stated: "Pulmonary involvement is especially characteristic of *B. haematobia* infections and is due to ova, worms and their toxins being filtered out in the pulmonary arterioles—reaching this destination from the pelvic plexuses of veins via the venacava."

In cases of human pulmonary schistosomiasis, Farid *et al.*[11] concluded that ova, or rarely adult worms, reached the lungs via the internal iliac veins (*S. haematobium*) or the portocaval anastamoses (*S. mansoni*). Richert and Krakauer[12] mentioned the possible passage of schistosome ova through vascular shunts in the liver. On the other hand, Bernard Shaw and Ghareeb[8] felt that the anastomoses between portal and systemic veins "normally seem insufficient to allow the passage of many parasites." In their opinion, it was only after progressive obstruction of the portal system and hyperplasia of the collateral circulation had occurred that conditions were favorable for pulmonary infection. Day[9] stated a similar view: "The gradual obstruction of the main portal channels in the liver from bilharzial cirrhosis leads to an opening of various anastomatic channels. There is a tendency for the venules under the capsule of the liver to become dilated and worms may be found close to the surface. Through these and other communications (retroperitoneal, etc.) ova and even worms may reach the lungs without having to pass through normal liver tissue or to travel by the inferior haemmorrhoidal plexus."

The latter postulates do not seem applicable to the lung shift of *S. mansoni* in mice following therapy. There is little reason to suspect that the conditions for pulmonary infection were any more favorable in the mice treated as described with tartar emetic or Miracil D than in the untreated controls. It seems more likely that anastomoses or shunts between the portal and systemic circulations are sufficiently numerous to allow the passage of mature worms at any time, but that such passages occur relatively infrequently unless a stimulus is provided (*i.e.*,

the introduction of an antischistosome compound into the host). It is not certain that the shunting process through such anastomoses is entirely passive, involving only dead or dying worms. Many worms found within the lungs of treated mice during the present studies were quite as active as those observed in the mesenteric veins of untreated controls, particularly during the first necropsy periods after dosage.

An interesting experiment which bears on the rapidity of passage of foreign objects from the liver to the lungs was performed by Prinzmetal *et al.*[15] A suspension of glass spheres was injected into the portal vein of rabbits within 5 mm of its entrance into the liver. Immediately thereafter the thoracic cavity was opened, and spheres of from 60 to 180 micra in diameter were recovered from the lungs of 10 out of 11 animals. The existence of non-capillary shunts between the liver and lungs was demonstrated, since the diameters of the spheres recovered were many times greater than the diameters of the capillaries.

The mean breadth of *S. mansoni* females is about 160 micra, and that of males several times greater.[16] It must be assumed, therefore, that non-capillary shunts in the livers of mice are fairly large, or, if not, the schistosomes may constrict somewhat during their passage. The latter condition would be more applicable to living worms.

A more difficult question to dispose of, even at the theoretical level, relates to the progression of only some of the worms beyond the liver during and after therapy. If the response were restricted to very heavy infections, it might be assumed that the liver became overcrowded and the excess worms were shunted into the lungs. Yet, pronounced lung shifts have been observed in relatively light infections. The shunting may be a random one, with widely divergent responses in different mice produced by differences in the deposition of worms within the liver in sites adjacent to or removed from possible passageways to the systemic circulation.

Possible analogies between mouse and human infections with *S. mansoni* in regard to a lung shift after therapy are at present purely conjectural. It is known that both ova and mature worms produce focal or widespread pulmonary lesions,[8, 9, 11, 12] and such lesions might be compounded if additional worms moved into the lungs as a direct consequence of certain regimens of therapy. However, the size and volume of the visceral organs of mouse and man differ enormously, as do the distances between the mesenteric and pulmonary vascular systems. Such factors may or may not influence the mass movement of schistosomes into the lungs during and after therapy. The possibility of lung shifts of schistosomes in man, and the extent to which such sequelae of treatment may alter pre-treatment pulmonary pathology, suggests further investigation.

## SUMMARY

Pronounced shifts of mature *Schistosoma mansoni* to the lungs of mice occurred during and following various regimens of therapy with tartar emetic and Miracil D, in contrast to the relatively few worms found in the lungs of untreated controls. The magnitude of these lung shifts sometimes equalled or surpassed the collateral hepatic shifts induced by therapy. Unpaired worms outnumbered paired worms in the lungs, and the ratio of males to females varied. Many dead worms were recovered from the lungs of treated mice from 2 to 4 weeks after the initial dosage.

The lung shift provides an additional criterion for evaluating the antischistosome activity of chemical compounds in mice, supplementing information derived from the usual examinations at necropsy of the mesentery, portal vein, and liver.

Implications of possible analogous responses to therapy in human schistosomiasis are discussed.

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# PHYSIOLOGICAL ACTION OF SCORPION VENOM

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The lethal scorpion of the Southwest, *Centruroides sculpturatus* Ewing, produces symptoms in vertebrates, including man, such as skeletal muscle convulsions, respiratory arrest, excessive salivation, mydriasis, visceral hyperemia, and gastric hyperdistention.[1] Many of these symptoms are similar to those produced by other lethal scorpions of the world.[2] However, there is little information available concerning the physiological actions of the *C. sculpturatus* venom.

Del Pozo[2] has described the pharmacological actions of venoms from several species of Mexican scorpions. He suggested that twitching of the skeletal muscles is produced by an effect of the venom on the motor end plate. Respiratory paralysis, the cause of death, resulted from a central neural action of the venom. Hypertension was found to be dependent on spinal pressor centers, release of pressor substances from the adrenal gland, and possibly direct action of the venom on the vascular tissue.

Diniz and Goncalves[3] have described a serotonin-like action of venoms from the Brazilian scorpions *Tityus bahiensis* and *T. serrulatus*, and Adam and Weiss[4] have identified serotonin chromatographically in venom of *Leiurus quinquestriatus*. However, no relationship between physiological activity and the serotonin component of the venom has been suggested.

The supposition that scorpion venoms stimulate the adrenal gland may be supported by Mohammed[5] who found that sublethal doses of Egyptian scorpion venom caused an elevated blood sugar level, and that the potassium concentration of the blood serum increased while the sodium level decreased.[6]

Results obtained from these studies have not been adequate to describe pharmacological effects of the venom of any particular species. Therefore, an investigation, first to survey the responses produced by the venom of *C. sculpturatus*, and second to determine the mode of action, was initiated in this laboratory.

## METHODS

*Preparation of venom.* A capillary pipette was used to collect venom following tetanization of the ejection musculature by the application of a faradic current to the postabdomen of the scorpion. Venom, collected in this manner from 1000 scorpions, was pooled, lyophilized, and stored in a deep freezer. Before injection, dried venom was weighed and diluted with water to produce a solution of known concentration. One milligram of venom per kilogram of body weight was injected into the femoral vein from a stock solution of 5 milligrams of venom per milliliter.

*Preparation of experimental animals.* Sodium pentobarbital, injected intravenously in dogs and rabbits and intraperitoneally in cats, was used as the anesthetic. The right femoral vein was cannulated to introduce venom and drugs. Adrenal glands of a group of eight cats were isolated from the circulatory system by ligating the adrenal veins and arteries. Frogs were lightly anesthetized with ether before the sciatic nerve was sectioned in the femoral region. Care was taken not to damage the circulatory system of the leg during this procedure.

*Experimentation.* Blood pressure was recorded either by a double membrane tambour or with appropriate transducers designed for use with the Grass Polygraph (Grass Instrument Co., Quincy, Mass.) and the Physiograph (E & M Instrument Co., Inc., Houston, Tex.). Respiration was recorded by means of a pneumograph or with an intrapleural cannula connected to an air pressure transducer. A balloon, fastened to the open end of a plastic tube and inserted into the duodenum, converted muscular contraction of the gut into pressure changes which were recorded by means of an air pressure transducer. Electrocardiograms were obtained through pin electrodes from the lead II position.

## RESULTS

The effect of venom on the respiratory rate was examined in four cats and two dogs in terms of the general effect on the ventilatory pattern. Of these animals, three cats and one dog developed an initial period of expiratory arrest following introduction of venom. This persisted from 20 to 30 seconds. The other dog exhibited

an increased rate followed by respiratory arrest (Figure 1) while the remaining cat had only a temporary increase in respiration. These animals, as well as all animals tested with venom, developed an intermittent gasping form of respiration which progressively diminished in amplitude and frequency until death resulted from anoxia.

Of the eight cats subjected to adrenal ligation prior to venom injection, four were tested for respiratory responses. Immediately after injection, one of these cats was found to have a decreased ventilatory activity while the other three remained normal. Later these animals developed characteristic respiratory abnormalities resulting in anoxia.

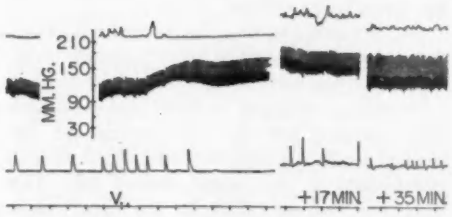


FIG. 1. Effect of scorpion venom (1 mg/kg) on skeletal muscle (upper trace), arterial blood pressure (middle trace), and respiration (lower trace) in a dog. Time interval: 5 seconds.

*Cardiovascular effects.* Table 1 summarizes effects of venom on heart rate and arterial blood pressure. Figure 1 illustrates action of venom on blood pressure of the dog. Typically, the initial elevation developed as an undulating surge of pressure which generally first exceeded and then dropped to a persistent hypertensive level within the first 3 minutes. This level remained constant until respiratory difficulties developed into the anoxic condition. Of the eight adrenal-ligated cats, two exhibited systolic pressure elevations of slightly over 25 mm Hg. (Table 2) while 11 of the 14 venom-treated normal cats exceeded this value (Table 1).

Hypertension was associated with vasoconstriction. Blanching of rabbit ears and viscera was observed after intravenous injection of venom. Similar phenomena were found in cats

TABLE 1

*Effect of scorpion venom on heart rate and blood pressure in anesthetized animals**

| Animals | Blood pressure (systolic/diastolic) | | | Heart rate (beats/minute) | | |
|---|---|---|---|---|---|---|
| | 0 Min. | 1–3 Min.† | Diff. | 0 Min. | 1–3 Min.† | Diff. |
| Cat 1 | 110/60 | 190/85 | 80/25 | 175 | 230 | 55 |
| 2 | 120/100 | 170/90 | 50/−10 | 184 | 250 | 66 |
| 3 | 145/130 | 215/130 | 70/0 | 200 | 200 | 0 |
| 4 | 150/125 | 180/120 | 30/−5 | 216 | 198 | −18 |
| 5 | 150/115 | 175/125 | 25/10 | 152 | 185 | 33 |
| 6 | 150/135 | 215/145 | 65/10 | 200 | 192 | −8 |
| 7 | 150/115 | 170/125 | 20/10 | 164 | 168 | 4 |
| 8 | 155/90 | 180/130 | 25/40 | 162 | 192 | 30 |
| 9 | 165/120 | 218/148 | 53/28 | 190 | 220 | 30 |
| 10 | 168/128 | 184/120 | 16/−8 | 200 | 200 | 0 |
| 11 | 170/140 | 250/175 | 80/30 | 156 | 210 | 54 |
| 12 | 175/120 | 270/180 | 95/60 | 168 | 188 | 20 |
| 13 | 180/160 | 200/150 | 20/−10 | 232 | 192 | −40 |
| 14 | 190/120 | 210/135 | 20/15 | 164 | 164 | 0 |
| Dog 1 | 130/90 | 170/120 | 40/30 | 152 | 172 | 50 |
| 2 | 150/120 | 170/125 | 20/5 | 130 | 200 | 70 |
| 3 | 185/155 | 210/165 | 25/15 | 164 | 168 | 4 |
| 4 | 185/120 | 200/130 | 15/10 | 148 | 175 | 37 |

* The amount of venom injected was 1 mg/kg body weight.

† Maximum rate reached during first 3 minutes after injection which was maintained until anoxia developed.

TABLE 2

*Effect of scorpion venom on heart rate and blood pressure of adrenal-ligated anesthetized cats**

| Animal | Blood pressure (systolic/diastolic) | | | Heart rate (beats/minute) | | |
|---|---|---|---|---|---|---|
| | 0 Min. | 1-3 Min.† | Diff. | 0 Min. | 1-3 Min. | Diff. |
| 1 | 120/60 | 145/70 | 25/−10 | 180 | 174 | −6 |
| 2 | 120/95 | 133/115 | 13/20 | 160 | 184 | 24 |
| 3 | 120/60 | 135/70 | 15/10 | 116 | 132 | 16 |
| 4 | 124/92 | 122/68 | −2/−24 | 140 | 160 | 20 |
| 5 | 128/100 | 155/105 | 27/5 | 150 | 180 | 30 |
| 6 | 145/110 | 140/112 | −5/2 | 150 | 170 | 20 |
| 7 | 175/105 | 180/118 | 5/13 | 160 | 160 | 0 |
| 8 | 190/140 | 180/130 | 10/−10 | 180 | 176 | −4 |

* The amount of venom injected was 1 mg/kg body weight.

† Maximum rate reached during first 3 minutes after injection which was maintained until anoxia developed.

and dogs. Right cervical sympathectomy of two rabbits failed to prevent vasoconstriction in the sympathectomized ears following administration of venom.

Heart rate changes varied from no change to an increase of 50 or more beats per minute or a decrease of as much as 40 beats per minute. In general, those cats and dogs with low initial heart rates showed more marked tachycardia than did those with high initial heart rates (Table 1).

Five of the normal cats, but none of the adrenal-ligated cats and none of the normal dogs, exhibited premature ventricular contractions intermittently during venom treatment. Cats exhibiting this phenomenon maintained a normal atrial rhythm but had a ventricular rhythm characterized by a short diastolic phase following a strong ventricular contraction. This resulted in an alternating series of high and reduced systolic pressure elevations. The short beat was accompanied by an inverted R spike. In addition, three normal cats exhibited temporary elevation of the T wave during the initial phase of blood pressure elevation.

*Effects on muscles.* Injection of 100 μg of venom into the dorsal lymph sac of frogs produced peripheral asynchronous skeletal muscle twitching. Eighteen envenomed frogs were divided into three groups of six each. The first group was brain pithed, the second double pithed, and the third group subjected to unilateral sciatic nerve transection. No reduction in the skeletal muscle response was found in the first group, while some twitching persisted in the second. The last group exhibited no twitching regions posterior to the level of transection. Since sciatic nerve transection prevented the response, the slight effect in the spinal pithed frogs is believed to have resulted from incomplete destruction of the spinal motor centers.

Another group of six frogs treated with tubocurarine chloride failed to produce muscle symptoms of envenomation. Injection of 10 units of tubocurarine, sufficient to block skeletal muscle and respiratory activity in the normal cat, also blocked venom induced twitching of the skeletal musculature of two venom treated cats. This treatment had no effect on the hypertensive condition.

By recording leg muscle activity in one dog (Figure 1) and by myographic recordings of two cats, evidence was found that venom caused an initial peripheral series of contractions. These observations showed that a brief series of contractions was succeeded by a quiet period before onset of muscle twitching.

Four isolated frog gastrocnemius-sciatic nerve-muscle preparations failed to develop twitching following addition of venom into the skin sac surrounding the muscle. Stimulation of the peripheral end of the cut sciatic nerve with a sufficient intensity to cause a twitch in the non-venom treated preparation failed to produce any abnormal response after addition of venom. In these preparations, doses of venom ranging from 50 to 200 μg of venom per muscle were used.

*Miscellaneous effects.* The effect of venom on intestinal motor activity was investigated in two cats and one rabbit. Normal peristaltic contractions were inhibited immediately after injection of venom. Direct observations of the viscera of cats supported these data. A response which developed gradually and did not become obvious until the animal neared death was the distention of the stomach with gas. This gas, which appeared to be carbon dioxide (since it failed to support combustion and had no odor), developed in the stomach and progressively extended through the pyloric sphincter into the small intestine. In severely bloated animals—particularly the dogs—

this action interfered with respiratory movements. Puncturing the stomach to relieve pressure facilitated respiration.

Other symptoms, such as salivation and mydriasis, appeared late in the sequence of reactions to the venom. Adrenal ligation had no influence on these responses.

## DISCUSSION

Little is known of the many effects produced by *C. sculpturatus* venom. In general, this substance produced a diphasic response in respiratory and skeletal musculature and to some extent in the cardiovascular system.

Respiratory difficulties developed in the following sequence: Initially, either an increased respiratory rate or respiratory paralysis occurred. After a period of no respiratory movement, a gasping form of respiration gradually developed. Skeletal muscles followed a similar pattern. Initially, brief contractions were found after intravenous administration of venom. This effect was succeeded by a quiescent period before characteristic muscle twitching appeared. It seems likely that the initial effects were due to a peripheral action of venom on muscle tissue while the continuous activity associated with gasping respiration and muscle twitching resulted from actions in central motor centers. Although no conclusions can be drawn concerning the exact site of venom action, the first effect seems likely to be a peripheral response since Del Pozo[3] found that intra-arterial injection of venom from Mexican scorpions produced contractions in muscle immediately after injection. The second effect on the skeletal musculature was found to be dependent on spinal motor pathways since brain pithing of frogs did not prevent this response, while sciatic nerve transection stopped the fibrillations in regions posterior to the level of transection.

Venom was found to have no significant direct effect on skeletal muscles. Twitching developed neither in venom-treated isolated frog gastrocnemius muscle preparations nor in lower leg regions of those frogs which had transected sciatic nerves. Also, no after effects were found following the application of threshold strength single shocks to the peripheral end of the cut sciatic nerve. Tubocurarine was found to suppress muscle twitching in both venom-treated frogs and cats.

Cardiac effects are difficult to interpret. Too much variability was found. Animals exhibited the following diverse effects: increase or decrease in heart rate, abnormal ventricular conduction leading to an inverted R wave, and elevated T waves. These responses require further study before conclusions may be obtained.

Hypertension was found to be a typical response of animals to venom. The possibility that vasoconstriction was produced by pressor substances rather than by sympathetic activity was suggested because sympathectomized rabbit ears developed vasoconstriction in the presence of venom. Two facts implicate the adrenal gland as the site of production of this pressor substance. First, hypertension is reduced in cats with isolated adrenal glands and, second, decreased intestinal motility accompanies introduction of venom into normal cats. The latter effect is similar to that of epinephrine on intestinal motor activity, and is in contrast to the direct action of venom on isolated rabbit duodenal strips. Increased activity of rabbit duodenal strips has been found (unpublished experiments) in vitro while gut motility is reduced in the intact envenomed animal.

Since some hypertension was found in adrenal ligated cats, the possibility exists that venom has either a direct vasoconstrictive effect or has an effect mediated by central pressor centers.

Although the nature of the adrenal hypertensive mediator is unknown, it seems likely it is an epinephrine-like substance. Epinephrine has the capacity to duplicate some venom actions such as vasoconstriction, inhibition of intestinal motility, production of mydriasis, piloerection, and perhaps salivation.

## SUMMARY

Venom of the scorpion, *Centruroides sculpturatus* Ewing, caused hypertension, respiratory failure, and skeletal muscle stimulation in anesthetized animals. No significant action of the venom was found on isolated skeletal muscle preparations. Hypertension resulted in part from release of pressor substances from the adrenal gland, although neither direct action nor action mediated by vasopressor centers can be ruled out. Respiration and skeletal muscle stimulation followed a pattern which suggests an initial peripheral action followed by an action dependent on the central nervous system.

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# OUT-PATIENT TOLERANCE AND ANTHELMINTHIC ACTIVITY OF NEW FORMULATIONS OF DITHIAZANINE

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During the past year a parasitosis clinic has been established by the Philadelphia City Health Department with the cooperation of the Communicable Disease Center. It is located in the new District 6 Health Building situated in the center of the city where most of the 20,000 Puerto Rican residents live. A diagnostic and treatment program has been provided primarily against schistosomiasis, amebiasis and nematode infections.

Parasitological surveys of Puerto Ricans in Philadelphia have revealed approximately 70% to be infected with *Trichuris trichiura*, 20% with hookworm, and 4% with *Ascaris lumbricoides*.[1] Dithiazanine (a cyanine dye, 3-3′ diethylthiadicarbocyanine iodide) was selected for routine use in the clinic for nematode infections in view of its broad spectrum anthelminthic activity reported by many investigators.[2-12] Initially several regimens using dithiazanine alone and in combination with tetrachlorethylene were employed. When it became apparent, however, that a considerable number of the patients receiving dithiazanine were experiencing unpleasant side reactions, several new formulations of the drug were prepared by the manufacturer, Eli Lilly and Company. The present paper presents a report of our experience with three of the new formulations in comparison with the standard commercial product and a placebo.§

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§ All of the dithiazanine products ("Delvex") and the placebo were generously supplied to the Parasitosis Clinic by Eli Lilly and Company, Indianapolis 6, Indiana. The use of these drugs does not constitute approval or endorsement by the Public Health Service and the Philadelphia Department of Public Health.

## MATERIALS AND METHODS

*Drugs.* All of the new formulations (Nos. II, III and IV) as well as the commercial product were in the form of enteric coated tablets which contained the same quantity of dithiazanine but differed as follows in the approximate lengths of their disintegration times in simulated intestinal fluid (pH 7.5): Dithiazanine (Commercial), 15 minutes; formulation No. IV, 45 minutes; No. III, 45 minutes; No. II, 60–90 minutes.**

The coatings (cellulose phthalate) for all of the formulations were identical and enabled them to remain intact in simulated gastric juice after several hours. The disintegration times in simulated intestinal fluid were dependent upon the composition of the cores of the tablets. The core of the commercial tablet was composed of dithiazanine iodide and starch, the cores of formulations II and IV contained methylcellulose, and formulation III, polyvinyl pyrrolidone. Dithiazanine IV differed from II in the size of the granules of the core ingredients. These formulations were the same as those similarly identified by Hammond and McCowen.[13]

The placebo was prepared to look like the other tablets, but of course did not contain the active ingredient.

The same dosage and course of therapy were used for each dithiazanine product. They were all given three times a day for 5 consecutive days according to the weights of the patients, the total daily doses being as follows: 20–30 lbs body weight, 200 mg; 30–40 lbs, 300 mg; 40–50 lbs, 400 mg; 50–60 lbs, 500 mg; 60 lbs and over, 600 mg. Patients assigned to the placebo group were given 1 tablet tid for 5 days.

The four dithiazanine products and the placebo were assigned to the patients at random in a consecutive order. The only exceptions were those few patients (heavily infected, debilitated,

** Information on the composition of the dithiazanine tablets and their disintegration times was supplied by Dr. James Hammond, Clinical Research Division, Eli Lilly and Company.

TABLE 1

*Prevalence of side reactions*

| | Dithiazanine | | | | Placebo |
|---|---|---|---|---|---|
| | D (S) | D IV | D III | D II | |
| Disintegration time (minutes) | 15 | 45 | 45 | 60–90 | — |
| Number of patients | 54 | 46 | 45 | 30 | 51 |
| Number patients with reactions | 33 | 7 | 8 | 2 | 2 |
| Per cent with reactions | 61 | 15 | 18 | 7 | 4 |

etc.) who it was considered for medical reasons should not chance the possibility of receiving the placebo. These patients have been excluded from the analysis. In like manner children who were unable to swallow tablets, unreliable individuals, persons of extreme age, and pregnant women in their third trimester of pregnancy were excluded from the study.

The patients were instructed to take the tablets 30 to 60 minutes after meals and were warned that their stools would have a blue coloration for several days. They were requested to return to the clinic the day after the end of therapy at which time the physician inquired about the occurrence of side reactions.

At the end of the specified follow-up period all of those patients which had been placed on the placebo were requested to return to the clinic in order to be treated with one of the dithiazanine formulations.

*Parasitological examinations.* The laboratory diagnosis and follow-up of the patients were primarily based upon the examination of normally passed stool specimens. Prior to therapy post-cathartic specimens†† were examined as a further check for amebae.

During the post-treatment follow-up period, specimens were requested at the end of therapy, and on the 15th, 30th and 90th day from the initiation of therapy. As would be expected, not all of the patients were able to adhere to this schedule.

All of the specimens were examined by direct wet mounts and by formalin ether concentration.[14] Egg counts were made by the standard smear technique of Beaver.[15] During the examination of the specimens the laboratory technicians were not aware of the specific medication received by the patients. The presence and absence of *T. trichiura* were used in the present evaluation of the new formulations of dithiazanine.

†† The cathartic "phospho-soda®" was supplied by the C. B. Fleet Co., Inc., Lynchburg, Virginia.

## RESULTS

Table 1 presents information on the occurrence of side effects in patients receiving the various products. The dithiazanine compounds are listed in order of increasing lengths of disintegration time. It will be noted that with the commercial product, 61% of the patients complained of annoying side reactions whereas only 15% and 18% of those receiving dithiazanine IV and III respectively had complaints. Those receiving dithiazanine II had fewer reactions (7%). Of the 51 patients receiving the placebo, 4% had complaints.

Table 2 records the types of side reactions reported by the patients. On the commercial tablets vomiting and nausea were the most frequent, with abdominal pain, diarrhea, chills, dizziness, etc. occurring less frequently. None of the patients receiving the new formulations of dithiazanine (II, III, IV) complained of nausea

TABLE 2

*Types of side reactions*

| Reactions | Dithiazanine | | | | Placebo |
|---|---|---|---|---|---|
| | D (S) | D IV | D III | D II | |
| Vomiting | 21* | 1 | 3 | 1 | |
| Nausea | 17 | | | | |
| Abd. pain | 10 | 3 | 4 | 1 | |
| Diarrhea | 4 | 3 | 2 | | 1 |
| Others | 6 | 1 | 1 | | 1 |
| Rx discont. | 1 | 1 | | | |

* Number of patients with indicated reaction.

TABLE 3

*Effectiveness against* T. trichiura

| Post-therapy follow up | Dithiazanine | | | | Placebo |
|---|---|---|---|---|---|
| | D (S) | D IV | D III | DII | |
| Number of patients | 30 | 33 | 35 | 26 | 42 |
| Number patients "cured" | 15 | 19 | 23 | 13 | 4 |
| Per cent "cured" | 50 | 58 | 66 | 50 | 10 |

although 5 of the 121 vomited at least once after taking the medication. On the whole, the side reactions were not serious and only with two patients was therapy discontinued, one receiving the commerical product and the other dithiazanine IV.

Table 3 presents information on the relative effectiveness of the various products as measured against *T. trichiura*. A "cure" indicates that the patient had at least two consecutive post-treatment examinations which did not reveal *Trichuris*. The last examination was performed between 1 and 4 months after the 1st day of therapy. The results demonstrate that the new formulations are as effective as the commerical product. The data would suggest that dithiazanine III is better than the others but at present these differences are not statistically significant. According to the criteria used, 10% of those receiving the placebo lost their infections.

The percentage reductions in egg counts of the patients on the various products essentially parallel the "cure" rates. Dithiazanine III had the greatest reduction (98%) while that for the other three dithiazanine formulations ranged between 74% and 88%. There was a 34% reduction with the placebo.

## DISCUSSION

Since the anthelminthic effectiveness of dithiazanine had been demonstrated convincingly by others, we were primarily interested in determining the relative tolerance of the various formulations. In our study a greater number of side reactions was recorded in patients on commercial dithiazanine than has been reported by others. Several of the other studies have been conducted in mental institutions where adequate histories are difficult to obtain. However, in the investigation by Wagner *et al.*,[16] with Mexican laborers, and in the one by Paravissini *et al.*,[17] with outpatient children in Puerto Rico, only about 30% of the patients complained of nausea and/or vomiting. Our rate with the commercial product, which is twice as high, cannot be explained at present.

The newer formulations were definitely better tolerated than the commercial dithiazanine. It would appear that the farther down from the stomach disintegration of the tablet occurs, the greater the chance that no reaction will occur. On this basis, dithiazanine II (or perhaps III) would be the drug of choice for infections by *T. trichiura*. However, since hookworm, *A. lumbricoides* and *Strongyloides stercoralis* are located in various portions of the small intestine, formulations with long disintegration times may not be as effective against them as the currently available commercial dithiazanine.

Other investigators have reported greater effectiveness of the commercial dithiazanine against *T. trichiura* than reported here. In some instances the dosage has been greater. However, in studies using the same standard dosage as ours, Swartzwelder *et al.*[4, 6] reported 97% of their patients cured and Paravissini *et al.*[17] reported 93.7% cured. A clear cut explanation for the apparent difference in effectiveness is not available, although the egg counts of Swartzwelder's patients were lower than those of ours and a concentration technic was not employed in the investigation of Paravissini. Young *et al.*[8] employed a concentration procedure ($ZnSO_4$) and had patients with greater average egg counts than ours had. Receiving 800 mg per day rather than standard 600 mg, 78% of their patients apparently lost their infections. These results are more in line with ours. Since the new formulation, dithiazanine II, is so well tolerated the possibility exists that the dosage can be increased considerably which may well result in greater effectiveness against *Trichuris* than has been reported to date.

Ideally a placebo would be expected to have no demonstrable effect on parasitic infections in the patient. However, there was a 34% reduction in the egg counts of *T. trichiura* in patients receiving the placebo, and 10% of these patients apparently lost this infection according to the criteria established for this evaluation. In a locality where little or no transmission is occurring, egg production and prevalence of *T. trichiura* would naturally undergo reduction with time. In Philadelphia the prevalence rate has been observed to drop from over 70% to 45% in Puerto Rican children after 6 years of residence in the United States.[1] In the present study the observed reduction of *T. trichiura* in the patients on the placebo may represent a combination of natural loss of worms and variations in laboratory performance. This reduction, regardless of cause, should be considered in appraising the observed effectiveness of the dithiazanine against *T. trichiura*. However, it does not directly influence

the primary objective of the present study which was to compare the relative effectiveness and tolerance of the four dithiazanine products.

SUMMARY

New formulations of dithiazanine with longer disintegration times than those of the present commercial product were found to be significantly better tolerated by Puerto Rican residents of Philadelphia treated as outpatients in a city clinic. The formulation with the longest disintegration time (60–90 minutes) was tolerated practically as well as the placebo.

The anthelminthic effect of the new formulations was observed to be equal to that of the present commercial product as measured against infections of *Trichuris trichiura*. Not enough infections of *Ascaris lumbricoides*, hookworm, and *Strongyloides stercoralis* were included in the study to determine the broad spectrum effect of the new formulations.

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# COMPARATIVE SENSITIVITY OF VIRUSES TO TREATMENT WITH DIETHYL ETHER AND SODIUM DESOXYCHOLATE*

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Two relatively simple chemical methods have been described for categorizing viruses: susceptibility to inactivation by bile salts and by diethyl ether. The history of the use of bile salts for the lysis of bacteria and for the inactivation of viruses is a venerable one that goes back more than fifty years. Smith,[1] who gives references to earlier work, found that sodium desoxycholate (SDC)‡ was the most active of the bile salts and that in a dilution of 1:1000 would inactivate influenza, louping ill and Rous sarcoma viruses, but was ineffective against ectromelia and vaccinia. A year later Burnet and Lush[2] extended Smith's observations to include ten viruses covering a rather wide range in size and pathogenesis. They found, as did Smith, that influenza and louping ill viruses were susceptible to the action of SDC as were also herpes, pseudorabies, Newcastle, myxoma and possibly fowl pox, while vaccinia, ectromelia and psittacosis were resistant. Theiler,[3] utilizing virtually the same procedure (treatment with SDC in a 1:1000 final dilution) found that all of 24 arthropod-borne viruses representing different sero-groups were sensitive to treatment, while GD VII and FA strains of mouse encephalitis, poliovirus, Coxsackie virus and the Mengo strain of EMC virus were not.

Though it had been observed that certain viruses, notably vaccinia and poliovirus, were resistant to the action of diethyl ether (DE), and that others, such as eastern and western equine encephalomyelitis, and St. Louis encephalitis viruses, were not,[4, 5] Andrewes and Horstmann[6] appear to have been the first to utilize DE susceptibility as a means of virus grouping. Their paper should be consulted for the range of such experiments and for the review of similar work by others. It will suffice to state here that representatives of the main virus "families" were examined either by the authors or by others for sensitivity to DE. A comparison of results reported on the sensitivity of viruses to treatment with SDC and DE reveals a striking similarity; the only discrepancy noted by Andrewes and Horstmann applied to psittacosis. They found it to be DE sensitive while Burnet and Lush reported it to be resistant to treatment with a 1:1000 dilution of SDC. However, these comparisons are based on work done by different investigators at different times.

The objective of the experiments here reported was to compare the relative sensitivity of a selected series of viruses to SDC and DE by running the tests in parallel on the same basic virus suspensions.

## MATERIALS AND METHODS

The 21 viruses examined comprised representatives of the arthropod-borne, entero- and respiratory (myxo- and adeno-) viruses. The methods for determining the inactivating effect of DE and SDC closely followed those described by Andrewes and Horstmann and by Theiler, except that 50% DE by volume was added to the virus suspension instead of 20% as recommended. However, the proportion of DE above the saturation point in the aqueous virus suspension (less than 10%) appears to be of no consequence.

As some of the viruses examined are not pathogenic to mice, tissue culture of appropriate cells or embryonated hen's eggs were employed for virus assay following treatment. For the most part the fluid of the infected tissue culture was used, after centrifugation at 10,000 rpm, as the

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‡ The abbreviation SD was used by Burnet and Lush and DCA by Theiler. SDC is chosen as a logical compromise between the two previous abbreviations.

TABLE 1

*Inactivating effect of treatment with sodium desoxycholate (SDC) and diethyl ether (DE) upon the enteroviruses*

| Virus | Method of virus assay* | Exp. No.† | Treatment with | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Sodium desoxycholate (SDC) | | | | | | Diethyl ether (DE) | | |
| | | | 1:200 dilution | | | 1:1000 dilution | | | | | |
| | | | Control virus titer | Treated virus titer | Log inactivated‡ | Control virus titer | Treated virus titer | Log inactivated | Control virus titer | Treated virus titer | Log inactivated |
| Polio 2 | RMK | 1 | 5.5 | 6.5 | −1.0 | 5.5 | 5.0 | 0.5 | 5.5 | 5.5 | 0 |
| | $TCD_{50}$ | 2 | 6.2 | 6.3 | −0.1 | 6.2 | 6.5 | −0.3 | 6.0 | 6.0 | 0 |
| | | 3 | 5.7 | 6.3 | −0.6 | 5.7 | 5.5 | 0.2 | 5.7 | 5.5 | 0.2 |
| | | 4 | 5.5 | 6.0 | −0.5 | 5.5 | 5.5 | 0 | 5.7 | 6.5 | −0.8 |
| Coxsackie A-9 | RMK | 1 | 7.0 | 6.5 | 0.5 | 7.0 | 6.5 | 0.5 | 6.5 | 6.0 | 0.5 |
| | $TCD_{50}$ | 2 | 6.7 | 7.2 | −0.5 | 6.7 | 6.7 | 0 | 6.7 | 6.5 | 0.2 |
| Coxsackie B-1 | RMK | 1 | 3.5 | 3.8 | −0.3 | 3.5 | 3.5 | 0 | 3.5 | 3.5 | 0 |
| | $TCD_{50}$ | 2 | 3.5 | 4.0 | −0.5 | 3.5 | 3.7 | −0.2 | 3.5 | 3.5 | 0 |
| ECHO 1 | RMK | 1 | 3.7 | 3.5 | 0.2 | 3.7 | 4.5 | −0.8 | 4.7 | 5.0 | −0.3 |
| | $TCD_{50}$ | 2 | 4.7 | 4.5 | 0.2 | 4.7 | 4.7 | 0 | 5.0 | 4.8 | −0.2 |
| | | 3 | 3.5 | 4.3 | −0.8 | 3.5 | 4.5 | −1.0 | 4.5 | 4.8 | −0.3 |
| ECHO 6 | RMK | 1 | 6.0 | 5.7 | 0.3 | 6.0 | 5.7 | 0.3 | 5.7 | 5.7 | 0 |
| | $TCD_{50}$ | 2 | 6.0 | 5.8 | 0.2 | 6.0 | 6.5 | −0.5 | 5.5 | 5.5 | 0 |
| ECHO 9 | RMK | 1 | 6.0 | 7.5 | −1.5 | 6.0 | 6.5 | −0.5 | 5.7 | 5.5 | 0.2 |
| | $TCD_{50}$ | 2 | 6.5 | 6.3 | 0.2 | 6.5 | 7.3 | −0.8 | 6.0 | 6.0 | 0 |

* RMK, $TCD_{50}$—Rhesus monkey kidney tissue culture cytopathic dose.

† Exp. No. indicates number of experiments performed.

‡ Minus sign (−) indicates higher titer in treated virus suspension.

basic virus suspension for testing. With some viruses which did not yield adequate titers in tissue culture, an infected mouse brain suspension in buffered saline containing 0.5% bovine albumin was substituted. For influenza viruses allantoic fluid of infected egg embryos in a dilution of 1:10 was used for the test suspension. Aliquots of the virus suspensions were treated with DE and SDC as outlined below.

*Diethyl ether (DE) treatment.* Equal volumes of the test virus suspension and DE were mixed in a test tube. The tube was tightly stoppered, agitated and stored in the refrigerator for 24 hours. The ether was then removed by evaporation in a vacuum jar to which a Hyvac oil pump was attached, and the de-etherized suspension titrated for virus. A control tube which contained an aliquot of the virus suspension but to which no ether had been added was handled in a similar manner.

*Sodium desoxycholate (SDC) treatment.* A 10% solution was prepared by dissolving desoxycholic acid in warm 0.3 M sodium hydroxide, thus converting the acid to the sodium salt. This solution was sterilized by filtration, then diluted 10 times in phosphate buffered saline containing 0.75% bovine albumin and kept refrigerated as the working SDC stock.§ On use the stock was warmed in a 37°C water bath to convert the cold gel to a liquid state; then the desired dilutions were made in phosphate buffered saline, pH 9.0. Equal amounts of SDC dilution and virus suspension were mixed and incubated for 60 minutes at 37°C and then titrated for virus infectivity. The control tube which contained equal parts of virus suspension and saline diluent was similarly incubated and titrated.

*Virus assay.* All tissue cultures used for virus titrations were prepared as previously described.[7] Cytopathic effects in fluid culture tubes or

§ This procedure in the preparation of SDC was followed because it was the one used by Dr. Delphine Clarke who prepared the SDC used by Dr. Theiler. However, the sodium salt can, if desired, be purchased directly from commercial concerns.

plaque formation under agar in 3-ounce bottles[8] were used as criteria for measuring virus infectivity. Four tubes or three bottles were inoculated with each dilution. The 50% tissue culture cytopathic dose ($TCD_{50}$) and the plaque-forming unit endpoint (PFU) were determined according to methods described by Reed and Muench[9] and Hsiung and Melnick,[10] respectively.

For the assay of the influenza viruses, 10 to 11-day old developing chick embryos were injected by the allantoic sac route with increasing log dilutions of virus. The embryos were incubated for 48 hours at 35–37°C, then chilled and the chorio-allantoic fluids withdrawn and tested for ability to agglutinate chicken red blood cells. Fifty per cent egg infectivity endpoints were determined according to the method described by Lauffer *et al.*[11]

## RESULTS

*Enteroviruses.* It was found that neither SDC nor DE had any inactivating effect upon the enteroviruses examined, including poliovirus 2, Coxsackie viruses A-9 and B-1, and ECHO viruses 1, 6 and 9 (Table 1). This applied to treatment with SDC in a dilution of 1:200 as well as in a dilution of 1:1000. In fact, there frequently appeared to be a rise in titer following treatment, especially with the more concentrated solutions of SDC. This was especially notable with poliovirus 2.

*Respiratory viruses.* Though differing markedly in their characteristics both influenza and adenovirus types have been placed in the respiratory virus category. Adenoviruses 1, 2 and 3 and influenza viruses A and B were examined. All three adenoviruses were found to be completely resistant to treatment with SDC in a dilution of 1:1000 and with DE (Table 2). Though not recorded in the table, adenoviruses were tested with a 1:200 dilution of SDC with no significant reduction in titer. On the other hand, both influenza A and B were markedly reduced in titer by both SDC (1:1000) and DE, although not completely inactivated by either.

*Arthropod-borne viruses.* Ten arthropod-borne viruses were examined, three belonging to serogroup A, three to group B, two to group C and two, Bunyamwera and California, to the ungrouped category. These viruses were selected not only because they represented different

TABLE 2

*Inactivating effect of treatment with sodium desoxycholate (SDC) and diethyl ether (DE) upon respiratory (Adeno and Myxo) viruses*

| Virus | Method of virus assay* | Exp. no.† | Sodium deoxycholate (SDC) 1:1000 dil. | | | Diethyl ether (DE) | | |
|---|---|---|---|---|---|---|---|---|
| | | | Control virus titer | Treated virus titer | Log inactivated‡ | Control virus titer | Treated virus titer | Log inactivated |
| Adeno 1 | Hela Cell $TCD_{50}$ | 1 | 2.5 | 2.5 | 0 | 2.5 | 2.5 | 0 |
| | | 2 | 2.5 | 2.5 | 0 | 2.0 | 2.5 | −0.5 |
| | | 3 | 3.0 | 3.0 | 0 | 3.3 | 3.5 | −0.2 |
| | | 4 | 3.3 | 3.5 | −0.2 | 3.5 | 3.5 | 0 |
| | | 5 | 3.0 | 3.5 | −0.5 | 2.5 | 2.5 | 0 |
| Adeno 2 | Hela Cell $TCD_{50}$ | 1 | 3.8 | 3.8 | 0 | 2.5 | 2.5 | 0 |
| | | 2 | 2.8 | 2.8 | 0 | 2.8 | 2.8 | 0 |
| | | 3 | 4.0 | 4.0 | 0 | 3.0 | 3.0 | 0 |
| Adeno 3 | Hela Cell $TCD_{50}$ | 1 | 1.8 | 2.0 | −0.2 | 2.5 | 2.5 | 0 |
| Influenza A | Embryonated egg | 1 | >6.5 | 1.5 | >5.0 | >5.5 | 0.7 | >4.8 |
| Influenza B | Embryonated egg | 1 | >6.2 | 2.8 | >3.4 | >6.5 | 2.2 | >4.3 |

* Hela Cell $TCD_{50}$—Hela Cell tissue culture cytopathic dose.

† Exp. No. indicates number of experiments performed.

‡ Minus sign (−) indicates higher titer after treatment than in control.

sero-groups, but also because they were isolated at widely distributed parts of the world, including North America, South America, Africa and the Far East. All, as will be noted in Table 3, were completely inactivated by both DE and a 1:1000 dilution of SDC.

It was found that chick embryo and hamster kidney cell cultures were particularly susceptible to the action of SDC, and it was therefore necessary to dilute the treated virus suspension tenfold in order to avoid the toxic effects associated with SDC. Consequently, if no virus growth occurred in the first tenfold dilution, the titer was recorded as less than (<) one log, and the log of virus inactivated recorded as greater than (>) the difference between one log and the titer of the control. Though not recorded in the table, a virus which was isolated from the brain of a nestling starling and tentatively designated as An 114, or Hilo virus,[12] was found to be susceptible to treatment with both SDC and DE. However, it is questionable if this virus, which has not yet been classified, belongs to either the arthropod-borne virus or the myxovirus groups.

In order to investigate the relative sensitivity of the arthropod-borne viruses to SDC, a selected number were treated with increasing two-fold dilutions of SDC (from 1:500 to 1:4000). It will be noted from the results plotted in Figure 1 that the most sensitive was West Nile belonging to sero-group B and inactivated by a dilution of 1:4000 SDC, followed by the two ungrouped

TABLE 3

*Inactivating effect of treatment with sodium desoxycholate (SDC) and diethyl ether (DE) upon arthropod-borne viruses*

| Virus | Serogroup | Method of virus assay* | Exp. no. | Treated with | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Sodium desoxycholate (SDC) 1:1000 dil. | | | Diethyl ether (DE) | | |
| | | | | Control virus titer | Treated virus titer | Log inactivated | Control virus titer | Treated virus titer | Log inactivated |
| EEE | A | CE | 1 | 5.0 | <1.0 | >4.0 | 4.5 | 0 | 4.5 |
| | | $TCD_{50}$ | 2 | 4.5 | <1.0 | >3.5 | 5.3 | 0 | 5.3 |
| | | | 3 | 5.0 | <1.0 | >4.0 | 5.0 | 0 | 5.0 |
| Sindbis | A | CE | 1 | 6.3 | <1.0 | >5.3 | 6.0 | 0 | 6.0 |
| | | $TCD_{50}$ | 2 | 5.7 | <1.0 | >4.7 | 6.3 | 0 | 6.3 |
| | | | 3 | 6.8 | <1.0 | >5.7 | 6.0 | 1.5 | 4.5 |
| Semliki forest | A | CE | 1 | 6.0 | <1.0 | >5.0 | 6.0 | 0 | 6.0 |
| | | $TCD_{50}$ | 2 | 5.5 | <1.0 | >4.5 | 5.7 | 0 | 5.7 |
| | | | 3 | 5.5 | <1.0 | >4.5 | 6.0 | 0 | 6.0 |
| Ilheus | B | RMK, TC | 1 | 2.8 | <1.0 | >2.8 | 3.6 | 0 | 3.6 |
| | | PFU | 2 | 2.7 | <1.0 | >2.4 | 3.0 | 0 | 2.0 |
| West Nile | B | HK $TCD_{50}$ | 1 | 3.5 | <1.0 | >2.5 | 3.5 | <1.0 | >2.5 |
| JBE | B | HK $TCD_{50}$ | 1 | 4.2 | <1.0 | >3.2 | 3.5 | <1.0 | >2.5 |
| Marituba | C | RMK, TC | 1 | 4.3 | <2.0 | >2.3 | 4.9 | 0 | 4.9 |
| | | PFU | 2 | 5.4 | <2.0 | >3.4 | 2.8 | 0 | 2.8 |
| Oriboca | C | RMK, TC | 1 | 2.0 | <1.0 | >2.0 | 2.3 | 0 | 2.2 |
| | | PFU | 2 | 3.2 | <1.0 | >3.2 | 3.4 | 0 | 3.4 |
| Bunyamwera | ungrouped | CE | 1 | 5.0 | <1.0 | >4.0 | 5.0 | 0 | 5.0 |
| | | $TCD_{50}$ | 2 | 3.5 | <1.0 | >2.5 | 3.0 | 0 | 3.0 |
| California | ungrouped | CE | 1 | 2.5 | <1.0 | >1.5 | 1.5 | 0 | 1.5 |
| | | $TCD_{50}$ | 2 | 5.8 | <1.0 | >4.7 | 5.3 | 0 | 5.3 |
| | | | 3 | 5.2 | <1.0 | >4.2 | 5.3 | 0 | 5.3 |

* CE $TCD_{50}$—Chick embryonated tissue culture cytopathic dose. HK $TCD_{50}$—Hamster kidney tissue culture cytopathic dose. RMK, TC, PFU—Rhesus monkey kidney tissue culture plaque-forming units.

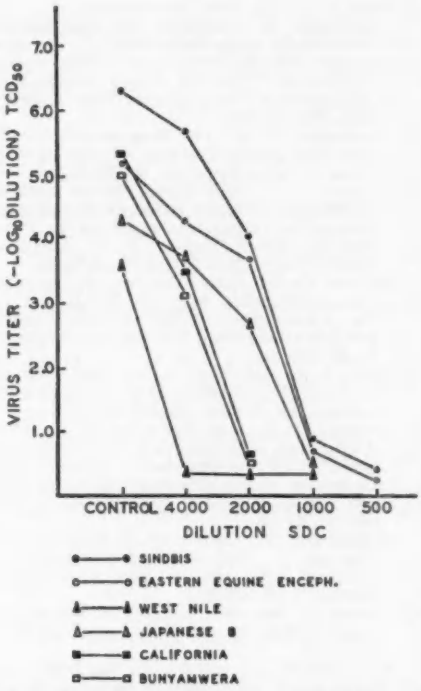


FIG. 1. The inactivation rates of several arthropod-borne viruses after treatment for 1 hr with varying dilutions of sodium desoxycholate.

sero-viruses, California and Bunyamwera, which were inactivated by a 1:2000 dilution. The A group viruses, EEE and Sindbis, were somewhat more resistant and required a 1:1000 dilution of SDC for complete inactivation.

## DISCUSSION

As initially stated, the principal objective of this study was to compare under analogous conditions the sensitivity of a selected number of viruses to sodium desoxycholate and diethyl ether, utilizing methods previously described. It was found that there was virtually complete accord between the two methods and that the results were consistent and reproducible. Thus, with the viruses examined either method may be used with the expectancy of obtaining the same result.

Each method has its advantages and disadvantages. The SDC method has the advantage of being more flexible in that relative sensitivity may be measured by using graded dilutions of the chemical, but its action is more likely to be interfered with by particulate matter or an excess of protein in the virus suspensions. Also, some tissue culture systems are quite sensitive to SDC, which prevents using tissue culture for detecting active virus in the undiluted suspension following treatment.

Diethyl ether has been used only in full aqueous saturation, and because of its volatility and low aqueous solubility it would probably not be practicable to use graded concentrations. Consequently, the only feasible way of assessing relative sensitivity is by varying the time of exposure and measuring the amount of virus inactivated in an aqueous suspension saturated with DE. However, DE has the advantage of being readily removable by reduced pressure following the treatment period.

If more were known about the process of virus inactivation by these chemicals, procedural modifications could be more logically investigated, and perhaps lead to improvement of the present methods. While this manuscript was being prepared, a note appeared[13] suggesting that the action of SDC is to strip off the outer protein coating of the virus particle and release the RNA, and that inactivation of virus infectivity is dependent upon the destruction of the released RNA, which in turn may depend upon whether or not the diluent used for titrating virus activity contains RNAase. This observation is worthy of further study as it may have a bearing upon standardizing the procedure of using SDC for virus grouping.

It is also worthy of note that Burnet and Lush found that sensitivity to saponin gave a clearer separation of viruses than did sensitivity to SDC, and it is suggested that this chemical be given consideration in future studies on this subject.

Ordinarily, the host and the nature of the specimen from which a virus is obtained will indicate the category or family to which the virus belongs; yet in certain instances this may be in doubt and preliminary screening with DE or SDC may be of practical assistance in categorizing the virus. The ultimate value of such chemical methods in the descriptive classification of viruses remains to be determined by more extensive and broader application.

SUMMARY

A comparison of the sensitivity of a selected number of viruses to treatment with diethyl ether (DE) and sodium desoxycholate (SDC) gave virtually identical results with each chemical.

Both DE and SDC inactivated all ten of the arthropod-borne viruses (representing serogroups A, B and C, and two ungrouped viruses) and two myxoviruses (influenza A and B). In contrast, poliovirus, two Coxsackie viruses, three ECHO viruses, and three adenoviruses were not inactivated by either DE or SDC treatment.

The application of these chemicals for categorizing viruses is briefly discussed.

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# NOTES ON ROCK HOLE BREEDING AND RESISTANCE OF *AEDES AEGYPTI* IN PUERTO RICO

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In Africa *Aedes aegypti* is both urban and sylvan in its choice of larval habitats, and the breeding sites in forested areas away from man on occasion may include rock holes,[1, 2] but in the New World the species is said to be almost exclusively urban, breeding in artificial containers in and around houses.[3, 4] Rock hole breeding was considered so uncommon in the Western Hemisphere that the literature seems to contain only one rather casual mention of the phenomenon,[5] and Horsfall[6] writes of tree holes, crab holes, etc., but not of rock holes, as natural habitats. Yet recent evidence indicates that rock hole breeding in the Americas is not rare and in fact may be locally important in a control campaign.[7] A detailed account of an instance is worthwhile not only because of the paucity of literature references but also because of the possible relationship to the manifestation of resistance presently occurring in the Caribbean Region.

The rock holes described in this study occur in a rough outcrop of limestone 75 to 90 feet above sea level directly adjacent to the Bay of Aguadilla. The terrain of the vicinity is mostly uneven with bare limestone protruding above the soil level in many places. Vegetation is of the scrub type and composed of grasses, vines and occasional small trees, as shown in Figure 6. The area is bounded on the west by the Bay of Aguadilla into which it drops sharply; on the north by vacant land with a sugar storage shed approximately 600 feet away; on the east by storage sheds and houses; and on the south by vacant land with rock outcroppings. The rock holes are located 80 to 300 feet from the nearest human habitation, and the distance from the nearest equipment storage shed is 100 to 200 feet.

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There are about two dozen rock holes of various dimensions in the area and they were examined on several different dates over a period of about 5 months (July to December 1959). Five were studied in detail, being designated as Rock Holes 1, 2, 3, 4, and 5 (shown in Figures 1, 2, 3, 4, and 5, respectively) and details of their characteristics on a date when many *aegypti* larvae were collected, 3 October 1959, are given in Table 1. Collections from the five rock holes were made as follows: Rock Hole 1—17 July 1959, 14 *aegypti*; 26 September, 31 *aegypti* and 44 *mediovittatus*; 3 October, 86 *aegypti* and 1 *mediovittatus*. Rock Hole 2—26 September, 2 *aegypti*; 3 October, 11 *aegypti*. Rock Hole 3—3 October, 11 *aegypti*. Rock Hole 4—3 October, 106 *aegypti*. Rock Hole 5—3 October, 9 *aegypti*. On 15 December 1959 Rock Holes 1, 2, 3, and 4 were dry. Rock Hole 5 contained water but no *aegypti* larvae. Samples of mud, dried scum, and debris from each of the dry rock holes were flooded with water in the laboratory.[8] The numbers of *aegypti* larvae noticed within 6 days after flooding the samples were as follows: Rock Hole 1, 0; Rock Hole 2, 0; Rock Hole 3, 8; and Rock Hole 4, 7. On 15 December eight rock holes which had not been examined previously were searched for larvae, and although water was present no *aegypti* were found; however, two larvae of *mediovittatus* were taken from one rock hole.

Two larval specimens preserved in balsam, each on a slide, were sent on 10 December 1959 to Dr. Alan Stone who confirmed the identifications and deposited them in the collection of the U. S. National Museum. They bear the following data on the labels: *Aedes mediovittatus*, Rock Hole A, Aguadilla, P. R., IX-26-59, A. Boike, I. Fox; *Aedes aegypti*, Rock Hole A, Aguadilla, P. R., IX-26-59, A. Boike, I. Fox (Rock Hole 1 = Rock Hole A).

Control operations in the general area were begun in May 1959 in the course of an *aegypti* survey of the Port Cities of Puerto Rico by the Division of Foreign Quarantine, U.S.P.H.S. Considerable breeding was found in and about

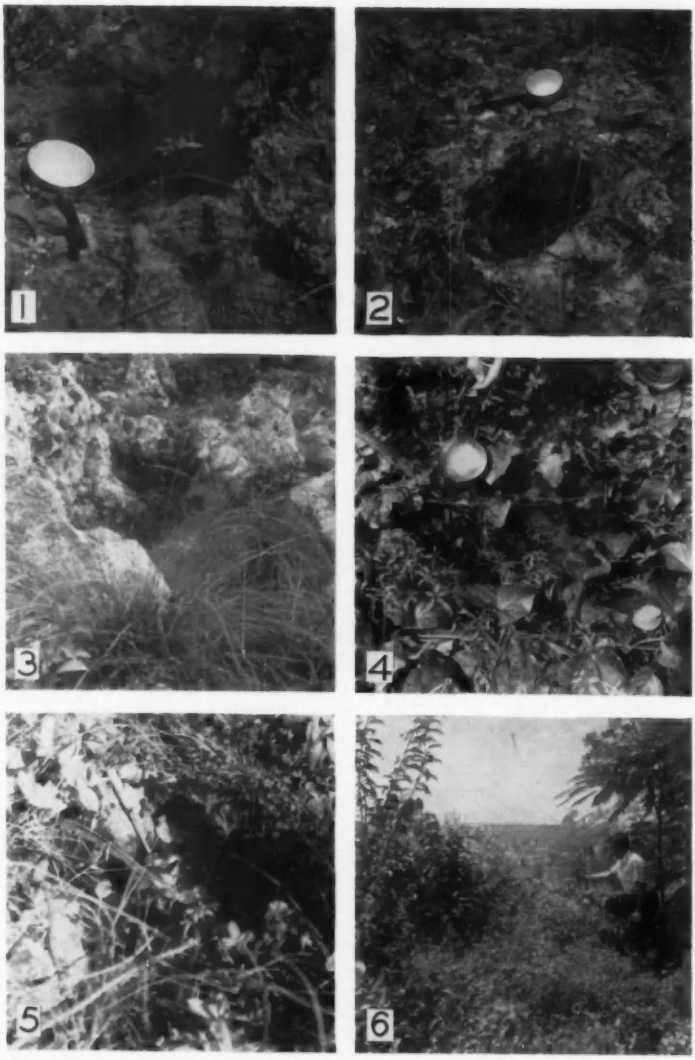


FIG. 1 to 5. Rock holes at Aguadilla, Puerto Rico, from which *Aedes aegypti* larvae were collected, and which were designated as Rock Holes 1, 2, 3, 4, and 5 respectively.

FIG. 6. View of vegetation around Rock Hole 1 with the dipper indicating the location of the rock hole.

(Photographs by Arthur H. Boike, Jr. on the following dates: Figures 1, 2, and 6 on 26 September 1959; Figure 3 on 19 August 1959; Figure 4 on 3 October 1959; and Figure 5 on 15 December 1959.)

the sheds near the rock holes in such artificial containers as commodes, toilet flush bowls, tires, tar drums, pails, wheel barrows, and tin cans. These breeding places were treated with dieldrin concentrate (15%) diluted at the ratio of one to three with water, and also efforts were made to eliminate some of the breeding places. Inspections were again made in June, July, August, and

TABLE 1

*Characteristics of rock holes in which* Aedes aegypti *larvae were breeding at Aguadilla, Puerto Rico, on 3 October 1959*

| | Rock hole | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 |
| Dimensions (inches) | 11 x 15 | 7 x 14 | 72 x 40 | 21 x 10 | 28 x 20 |
| Maximum water depth (inches) | 4.5 | not done | 9 | 2.25 | 2 |
| Nature of bottom debris | organic material | organic material | stone, plants, roots | organic material | sandy |
| Temperature (°F) under water surface | 79 | not done | 89 | 88 | not done |
| Temperature (°F) at bottom | 78 | not done | 83 | 81 | 92 |
| pH | 7.6 | not done | 7.6 | 7.6 | 7.6 |
| Feet from nearest human habitation | 270 | 130 | 180 | 80 | 300 |
| Feet from storage shed | 160 | 200 | 170 | 100 | 170 |
| No. of larvae collected | 86 | 11 | 11 | 106 | 9 |

November, and each time larvae were found in artificial containers which were treated with 50% DDT dust. However, no control measures were directed against the rock holes described in this paper. *Aegypti* was not eliminated from the general area despite these treatments with dieldrin and DDT over a period of several months. The three most important factors in the control failure were believed to be the presence and recurrence of many artificial containers providing breeding places in great abundance; the persistence of breeding in rock holes hidden by dense vegetation (Figure 6) and overlooked in inspections; and the high resistance of the strain to dieldrin and DDT.

Inasmuch as rock hole breeding is said to be a rare occurrence in the Western Hemisphere, the possibility that the phenomenon indicated behavioristic resistance had to be considered. According to some authorities a necessary element of proof of behavioristic resistance "would be for populations to appear sensitive in the toxicological test."[9] However, *aegypti* larvae originating from the rock hole specimens proved to be highly resistant in laboratory tests. It was concluded then that the rock hole breeding was the result of great population pressure and the proximity of the rock holes to the more usual habitats. Nevertheless, certain aspects of this problem make further investigations in the Caribbean Area necessary; for example there is the possibility that the segment or strain of the *aegypti* population which frequents households is being killed off by insecticides, leaving that portion or race which breeds readily in natural habitats and which therefore may become the dominant strain.[10]

Larval resistance was ascertained with W.H.O. Test Kit No. 259 solutions of DDT, dieldrin, and lindane. Specimens collected as larvae from Rock Holes 1 and 4 on 3 October 1959 were the parents of a laboratory colony which provided on 24 November 1959 eggs of the $F_3$ generation from which developed the larvae used in the tests completed on 4 January 1960. To make the tests 20 fourth-instar larvae were exposed for 24 hours without food in 200 ml of insecticide solutions at a concentration of 2.5 parts per million in open half-pint cardboard containers having a depth of two inches and a diameter of three and three-eighths inches. Dead and dead-plus-moribund larvae were counted separately to calculate mortality percentages because it was not possible to distinguish accurately moribund from living larvae. Two replicates were made with appropriate controls and the results are shown in Table 2. Since the $LC_{50}$ of *aegypti* larvae of DDT-susceptible strains is said to be below 0.01 parts per million[11] and 100% mortality should normally occur at 0.02 ppm of DDT or dieldrin,[12] it is clear that the specimens from Aguadilla rock holes were highly resistant to all three insecticides, more particularly so to dieldrin, for 100% mortality did not result at even 2.5 ppm. The laboratory experiments therefore confirmed the

TABLE 2

*Per cent mortality of* Aedes aegypti *fourth stage larvae after 24 hours of exposure to 2.5 ppm of DDT, dieldrin, and lindane (range of at least two replicates, dead only and dead plus moribund) from various localities in Puerto Rico*

| Locality | DDT | | Dieldrin | | Lindane | |
|---|---|---|---|---|---|---|
| | D | D + M | D | D + M | D | D + M |
| 1. Isla Verde (Lab. Colony, $F_{30\pm}$) | 40–75 | 40–76 | 7–18 | 11–27 | 70–96 | 74–96 |
| 2. Aguadilla (Rock Holes, $F_2$) | 64–84 | 76–82 | 0–4 | 4–4 | 52–68 | 72–80 |
| 3. Santurce (From Field) | 43–68 | 84–91 | 35–40 | 42–80 | 60–70 | 75–80 |
| 4. Santurce ($F_1$) | 55–65 | 70–70 | 35–40 | 60–70 | 90–100 | 95–100 |
| 5. Old San Juan ($F_3$) | 70–75 | 100–100 | 16–16 | 44–50 | 75–89 | 100–100 |
| 6. Near Ft. Buchanan ($F_2$) | 85–100 | 100–100 | 15–21 | 57–63 | 85–100 | 100–100 |

field observation of control failure due to resistance to insecticides.

The results of these toxicological tests were expected because previous studies had demonstrated a highly resistant strain of *aegypti* in Puerto Rico. In 1956 there was a recrudescence of population in the city of San Juan and in the vicinity of the International Airport in Isla Verde.[13] A colony was established from larvae and adults taken mostly from a concrete tank at Boca de Cangrejos, Isla Verde, in November 1958. This colony, which has been inbreeding continuously, provided specimens for experiments completed in 1959 which demonstrated that the larvae of the Isla Verde, Puerto Rico, strain were highly resistant to DDT, lindane, dieldrin and chlordane and moderately resistant to malathion, dipterex and diazinon but, relatively speaking, showed susceptibility to Bayer 21/199.[14] Table 2 gives the mortality range of six replicates after the colony had been maintained for almost a year and was at the approximate $F_{30}$ generation in September and October 1959. It will be seen that the Aguadilla rock hole material did not differ markedly from the Isla Verde laboratory strain as regards resistance to DDT, dieldrin and lindane.

For the purpose of comparison colonies originating from different localities in Puerto Rico were also tested. To obtain a sufficient number of individuals for the tests it was necessary in most cases to breed colonies to the $F_2$ generation. The following localities provided material for the tests whose results are shown in Table 2: (1) Isla Verde (see above). (2) Aguadilla rock holes (see above). (3) Santurce, larvae collected from a pail on 14 January 1960 and tested on 15 January 1960. (4) Santurce, larvae collected from a pail on 13 October 1959, colonized; and the $F_1$ generation eggs of 24 November 1959 reared to larvae and tested on 12 January 1960. (5) Old San Juan, larvae collected from a gutter in a yard on 30 September 1959,colonized; and the $F_3$ generation eggs of 30 November 1959 reared to larvae and tested on 19 January 1960. (6) Near Fort Buchanan, larvae collected from tires on 17 September 1959, colonized; and the $F_2$ generation eggs of 17 November 1959 reared to larvae and tested on 18 January 1960. These strains all showed definitely high resistance to dieldrin and most also indicated unequivocal resistance to DDT and lindane. The experimental results for the most part represented laboratory confirmation of control failure due to resistance to DDT and dieldrin. Since different replicates may vary greatly, the 100% dead-plus-moribund mortalities of the Old San Juan and Near Fort Buchanan strains are not considered significant. As regards Old San Juan, larvae taken from the same habitat on 8 September 1959 were tested directly with 2.5 ppm DDT with the following 24 hour percentage mortalities in two replicates: dead only, 31 to 41; dead plus moribund, 38 to 41. Therefore, Old San Juan must be included among the

localities which show resistance to both DDT and dieldrin, and this is in keeping with the control experience. The distance from Isla Verde to Aguadilla along the north coast is approximately 90 miles. Since the toxicological data are similar for both places, it is reasonable to assume that *aegypti*, if it is not already resistant to DDT and dieldrin along the whole north coast, will quickly become so. The continued use of these insecticides there will be in vain.

Resistance of *aegypti* to both dieldrin and DDT apparently was first noted in Puerto Rico.[14] Previously, writers had suggested that these two insecticides are associated with distinct types of resistance having separate toxicological, biochemical, and genetical natures, and that one type, such as DDT resistance, does not involve the other type, dieldrin resistance.[13] It does not seem possible to reconcile this idea with the evidence from Puerto Rico and radical modification or even complete abandonment of this idea is suggested.


## SUMMARY

Either by the finding of larvae or by the flooding of dry material, natural rock holes in Aguadilla, Puerto Rico, were shown to be breeding *Aedes aegypti* on four occasions during a period of 5 months, 17 July to 15 December 1959. The rock holes were photographed and some of their characteristics recorded. Since the area had resisted control measures involving treatment with DDT and dieldrin, larvae from the rock holes were used to establish colonies which were subjected to toxicological tests in the $F_3$ generation. The larvae were found to be highly resistant to DDT, dieldrin, and lindane. At 2.5 ppm the 24 hour percentage mortality of two replicates, counting dead-plus-moribund larvae, ranged from 76 to 92, 4 to 4, and 72 to 80 respectively; and counting dead only, the percentage mortalities ranged from 64 to 84, 0 to 4, and 56 to 68 respectively. Control failure in the area was ascribed to the abundance of artificial containers, the hidden, inaccessible rock hole breeding places, and the resistance of the strain to DDT and dieldrin. Results were compared with those from tests on larvae from five other localities. It was concluded that *aegypti* along the whole north coast of Puerto Rico is resistant to both DDT and dieldrin, or will shortly become so, so that continued use of these insecticides is believed to be of little value. The abandonment of the current idea that *aegypti* populations do not show resistance to both DDT and dieldrin is also suggested.



## ACKNOWLEDGMENTS

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# STUDIES OF SCHISTOSOMIASIS IN PRIMATES: OBSERVATIONS ON ACQUIRED RESISTANCE (PROGRESS REPORT)

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The U. S. Army Tropical Research Medical Laboratory and the University of Puerto Rico, School of Medicine and School of Tropical Medicine, have undertaken jointly a long-term investigation of schistosomiasis mansoni in the primate, *Macaca mulatta*. This report is concerned with observations related to acquired resistance resulting from infection. The main topics of consideration include fecal egg-counts during the immunizing period and following the first massive challenge, and numbers of worms and their state of maturity at varied intervals after a second challenge.

It has been found repeatedly that acquired resistance occurs in mice following infection with different species of schistosomes, but the resistance is only partial.[1-4] In contrast, certain primates have exhibited near-complete resistance after initial infection(s). The investigation of Vogel and Minning[5] conducted on numerous rhesus monkeys clearly demonstrated strong resistance against *Schistosoma japonicum* after about 2 years of infection. Studies using *S. mansoni* have given similar results, but fewer monkeys were involved in the tests. Meleney and Moore[6] obtained evidence of acquired resistance in one monkey (*Pithecus mordax*) which was immunized with a bisexual infection of *S. mansoni* and challenged with the same species. Standen[7] obtained resistance in two *Macacus rhesus* monkeys, as indicated by their tolerance of severe challenges and absence of egg production thereafter (see also personal communication to Fairley).[8] Cram and Files[9] noted spontaneous cure of *S. mansoni* and subsequent resistance in monkeys, as shown by egg counts. Fairley *et al.*[10] and Kagan,[11] using other schistosomes for which their monkeys had a strong natural resistance, found that the brief life span of the worms was reduced further in the case of challenge infections.

Newsome,[12] and Vogel and Minning[5] were confident that acquired resistance occurs in man, but Fairley[8] was much less certain. Indeed the evidence for man is largely circumstantial and epidemiologic in nature, except for the investigations of Fisher[13] who noted in two highly endemic African villages that even when males of ages 5 to 30 years had *S. intercalatum* infection rates of 72 and 79 per cent, occurrence in the older age groups was 0 and 4 per cent; moreover, adults showed infection rates equal to those in the younger groups where the endemicity was of a lower level. This evidence of acquired resistance was further supported by well-controlled challenges in six older volunteers from a highly endemic village. Eggs were not recovered after 3- and 6-month intervals following exposure, and although a few were obtained from three of the volunteers after 8 months, their occurrence was brief.

Although much work has been done on the immunology of schistosomiasis, it has been emphasized by Newsome[12] that even basic features of resistance and the mechanism involved are still poorly understood. Even though resistance through vaccination may never prove feasible, immunologic information is important in the development of sound concepts of therapy and prevention.[8, 12]

## METHODS

Twenty-six monkeys, the original group used in this study, were all *Macaca mulatta*. They

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were young, weighing 1.8 to 2.7 kg, and in good physical condition. One ultimately became paralyzed, but survived the experiment. Tattooing was used to insure identities of the monkeys.

Three plans of exposure were imposed on the experimental animals in the attempt to induce resistance: Group I, a single exposure to 1000 cercariae; Group II, combinations of moderate (500 cercariae) and multiple light exposures (25 to 50 cercariae); and Group III, a series of 26 light exposures (25 to 50 cercariae), given at 35 day intervals.

Group I was comprised of seven monkeys. One of these (No. 34) died early in the experiment, before resistance was acquired, and was transferred to the lethal-exposure control group. Another monkey (No. 28) became serologically positive after initial exposure, but failed to discharge eggs. It was re-exposed to 1000 cercariae after 450 days and developed a "normal" infection. This animal was retained in Group I. The 11 monkeys comprising Group II all received an initial exposure to 500 cercariae. Four were infected a second time with 500 cercariae after 250 days, and received 20 light exposures between days 500 and 1200 (Nos. 9, 18, 22, and 26; No. 18 died). Two animals received only the two moderate exposures to 500 cercariae (Nos. 19 and 27). The remaining five monkeys did not receive the second lot of 500 cercariae, but in turn were given the 20 light exposures (Nos. 1, 2, 11, 13 and 21; Nos. 2 and 11 died). Group III (26 light exposures) was comprised of six monkeys (Nos. 3, 4, 24, 30, 31 and 32; No. 4 died).

Two previously uninfected monkeys, completing the total of 26 in the series, were used as lethal-exposure controls. They were exposed to 5000 cercariae and died of their infections 54 and 56 days later. Monkey 34 of Group I which died 64 days after exposure to 1000 cercariae was included with these two controls.

Exposures to 5000 cercariae were used as challenges. For reason of caution three animals of Group I were given sublethal challenges of 500 cercariae at 700, 800 and 900 days after initial exposures, but thereafter only the massive exposures were used. Each monkey received two of the massive challenges with an intervening period of 85 to 245 days. The former period was judged the shortest acceptable for detecting a recurrence or an increase in the number of *S. mansoni* eggs. After the second challenge the animals were sacrified at varying intervals and observations were made on the development and the life span of the worms in the resistant hosts. The mesenteric veins and the liver were perfused separately by the method of Radke, Berríos-Durán and Moran.[14]

Cercariae from numerous snails were pooled and quantitated by means of an automatic micro-pipette with a selector collar. When the monkeys were exposed to large numbers of cercariae, they were strapped belly down on an exposure board and cercarial baths were applied from below. For light exposures, cercariae were dropped onto shaved skin. A period of 45 minutes was allowed for penetration, and the infectivity of the cercariae was verified on each occasion by exposing five mice.

Blood samples were taken from each animal before exposure and at weekly intervals throughout the experiment. The sera were used for cercarial agglutination, circumoval precipitation and complement fixation tests with egg, cercaria and adult antigen. The results of this phase of study will be reported at a later date. Stools were obtained twice a week and a count was made of all eggs recovered from 2 grams of feces by the AMS III ether sedimentation technic.[15]

## RESULTS

*Egg recoveries.* During the immunizing period for monkeys of *Group I*, near-maximum egg production persisted for only 60 to 90 days, occurring between 42 and 150 days after exposure. The subsequent decline in the number of eggs was sharply defined, reaching a low level by 200 days and becoming negligible within 300 days. Counts recorded for Monkey 8, shown in Figure 1, illustrate the typical pattern of egg production.

Three sublethal challenges of 500 cercariae, given at 700, 800 and 900 days to three monkeys in Group I, did not elicit an increase or a renewal of egg recovery from the feces. When these and the other three members of the group were exposed to the maximum challenge of 5000 cercariae, after about 1000 days, there was again no increase or renewal of eggs (Figure 1).

For *Group II*, the results were so variable that a single animal could not be selected as representative; moreover, factors other than exposure pattern seemed to influence results. Five animals have been included in Figure 2 to indicate the

nature of the findings. Although Monkeys 9, 26 and 22 were exposed on the same day, egg production was low for the first two, whereas for Number 22 it was very similar to that observed in animals exposed to 1000 cercariae, but with somewhat smaller numbers. In Monkeys 9 and 26, the second exposure to 500 cercariae elicited a slight increase in the number of eggs, while for Monkey 22, the second exposure appeared merely to sustain egg counts for a brief period. When Monkeys 9 and 26 were given the series of light exposures, the former discharged no eggs, but a slight increase occurred in the latter. The results suggest that the pattern of natural resistance for Monkeys 9 and 26 were similar at the beginning of the experiment, but quite different from that of Monkey 22; however, egg production was suppressed in Monkey 22 as well as in the other two animals after a challenge exposure to 5000 cercariae.

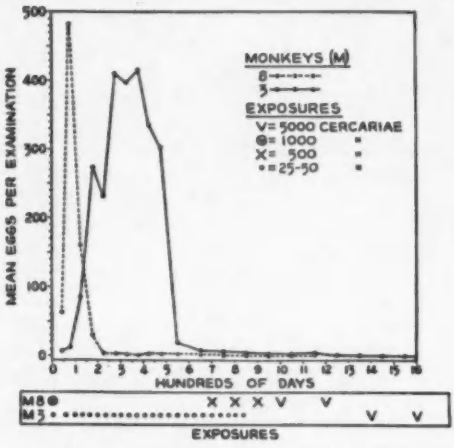


FIG. 1. Mean egg recoveries, based on total eggs obtained from 10–14 stool examinations (AMS[III] method) per 50-day interval, from two monkeys (Nos. 8 and 3) given respectively one heavy and numerous light immunizing exposures followed by heavy challenging exposures.

Monkeys 1 and 13, which received a single exposure to 500 cercariae initially, discharged only a few eggs and then responded quite differently when they were given a series of light exposures. Both animals showed marked increases in the number of eggs, but for Monkey 13 the number became negligible before the completion of the series. On the other hand egg production in Monkey 1 rapidly reached a high level and this high level was maintained until the animal was sacrificed 800 days later. The pattern of egg production for this animal resembled that of

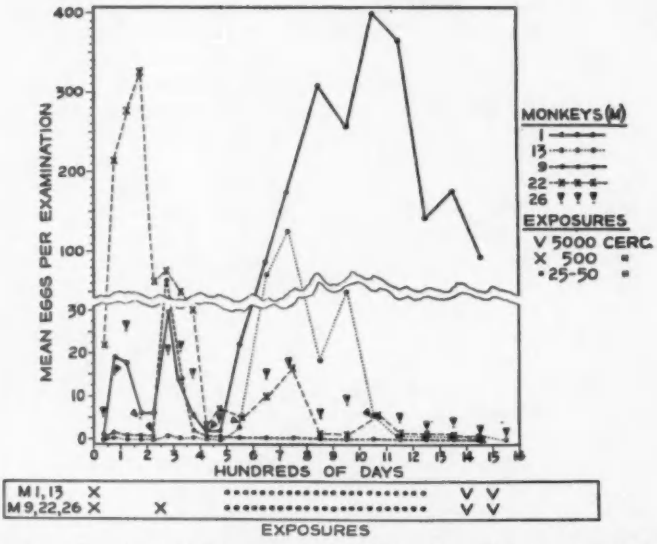


FIG. 2. Mean egg recoveries, based on total eggs obtained from 10–14 stool examinations (AMS[III] method) per 50-day interval, following combinations of moderate and light immunizing exposures, and after challenge.

Monkey 3 in Group III (Figure 1). It is apparent that the light infection acquired by these animals from their initial exposure had not conferred resistance within the 500 days preceding the series of light exposures.

There was no increase or renewal of egg recovery from monkeys of Group II following challenge with 5000 cercariae.

*Group III* animals, receiving 26 light exposures, all showed similar egg production patterns during the immunizing period, but differed as to the number of eggs produced. The record of Monkey 3, selected to represent the group, is shown in Figure 1. Egg numbers generally reached a near-maximum level after 200 days, or before one-third of the exposures had been completed, and remained high with some fluctuation for a period of about 300 days. A sharp, in some cases precipitant, decline in eggs followed, and then egg counts were negligible during the interval encompassing the last third of the exposures. As in the other two groups, lethal challenges with 5000 cercariae had no effect on egg numbers.

*Worm recoveries following a second challenge exposure.* It has been shown in the previous section that the first lethal challenge did not cause death of resistant animals, and the egg counts indicate that the worms resulting from these exposures did not mature. Autopsies of animals, made at intervals of 6, 15, 22, 42, 55 and 65 days after a second challenge, furnished information on the fate of new and pre-existing worms in the resistant host. Initially, periods ranging up to 245 days were allowed between challenges but later it was felt that an interval of 85 days was sufficient.

In three of the animals (Nos. 6, 8 and 9) sacrificed 6 days after the second challenge with 5000 cercariae, no worms were obtained by perfusion of the portal system (Table 1). In these animals all of the worms from the first challenge and previous exposures had been eliminated, and those from the second challenge had not had time to reach the liver and mesenteric vessels. However, histologic studies of the lungs revealed significant cellular reactions. A report on these and other histo-pathological findings will be published separately. In two animals (Nos. 19 and 22) autopsied on the 15th day after the second challenge, only schistosomules and small worms were found in the portal circulation. The number found accounted for about 2% of the number of cercariae used in the second challenge.

TABLE 1

*Worm recoveries of* S. mansoni *at varied intervals following second challenge with 5000 cercariae*

| No. of animal | Days between massive challenges | Days between 2nd challenge and sacrifice | Size and no. *S. mansoni* recovered | | | |
|---|---|---|---|---|---|---|
| | | | Schistosomules | Small sizes | Mature sizes | Totals |
| 6 (I) | 168 | 6 | 0 | 0 | 0 | 0 |
| 8 (I) | 132 | 6 | 0 | 0 | 0 | 0 |
| 9 (II) | 91 | 6 | 0 | 0 | 0 | 0 |
| 19 (II) | 98 | 15 | 58 | 28 | 0 | 86 |
| 22 (II) | 85 | 15 | 94 | 2 | 0 | 96 |
| 23 (I) | 245 | 22 | 275 | 146 | 27 | 448 |
| 1 (II) | 111 | 23 | 151 | 322 | 21 | 494 |
| 21 (II) | 105 | 42 | 87 | 416 | 0 | 503 |
| 33 (I) | 85 | 55 | 0 | 1 | 0 | 1 |
| 27 (II) | 84 | 65 | 0 | 831 | few | 831* |
| 28 (I) | 84 | 65 | 0 | 865 | few | 865† |
| 29 (I) | 183 | 65 | 0 | 0 | 12 | 12 |

*Lethal-exposure control monkeys*

| | No. cerc. | Days survival | No. worms | | | Per cent |
|---|---|---|---|---|---|---|
| 34 | 1000 | 64 | 0 | 0 | 514 | 51 |
| 35 | 5000 | 54 | 0 | 0 | 721 | 14‡ |
| 36 | 5000 | 56 | 0 | 0 | 1468 | 29 |

( ) Roman number in parenthesis designates exposure group.

* 50,000 cercariae used in second challenge instead of 5000.

† This monkey was slow in suppressing the immunizing exposure.

‡ Monkey refrigerated overnight—recovery may have been incomplete.

Two animals (Nos. 23 and 1) which had continued to discharge some eggs throughout the experiment were sacrificed on the 22nd and 23rd days and the adult worms found in these animals were commensurate with earlier exposures involving 6250 and 7500 cercariae. About half of the worms from the second challenge were schistosomules, and these and the small forms accounted for about 10% of the cercariae in the final challenge. All of the worms found in one monkey (No. 21) 42 days after a second challenge were schistosomules or individuals that were much smaller than could be expected from previously unexposed animals. That these worms could not have developed and produced eggs was evident from the results of the first challenge,

given 147 days prior to sacrifice; *i.e.*, no eggs were discharged and no mature worms were recovered. From one monkey (No. 33) sacrificed at 55 days after a second challenge, only one small worm was recovered. Although over 800 small worms were found in two of three animals sacrificed at 65 days (Nos. 27 and 28), their presence in these animals could have been accounted for by unusual circumstances. Number 27 had received a second challenge with 50,000 cercariae rather than 5000 (to facilitate pathologic observations) giving a recovery rate of less than 2%; and Number 28 had been relatively slow in suppressing the egg production of the immunizing infection which had developed from exposure to 1000 cercariae. The third monkey (No. 29), sacrificed at 65 days, was found to have only 12 worms. From Monkey 9, which was sacrificed 6 days after the second challenge and 97 days after the first, no worms were recovered.

## DISCUSSION

The present investigations and those of Vogel and Minning[5] were conducted along somewhat similar lines. *Macaca mulatta* monkeys were used in both studies, but the species of schistosome used in the experiments differed, being respectively *S. japonicum* and *S. mansoni*. Vogel and Minning recorded very complete histories of immunization, egg recoveries and acquired resistance for 16 animals among a group of at least 22. Of these, 10 received bisexual immunizing infections. We immunized 24 monkeys with bisexual infections, but 5 of them died.

Only our Group III which received multiple light exposures has a true counterpart in the earlier investigation of Vogel and Minning.[5] Four of their monkeys were so exposed, while three others received two or more exposures to varied numbers of cercariae (bisexual) at irregular intervals. The latter are not unlike certain of our Group II animals, particularly those receiving only two immunizing exposures to 500 cercariae. The other 3 of their 10 monkeys which received bisexual immunizing infections were sacrificed before resistance might be expected to occur and, therefore, afforded observations on normal rates of worm recoveries, which were as follows: 17% of 403 cercariae (Monkey Negus), 16% of 1512 cercariae (M. Pieter) and 30.5% of 800 cercariae (M. Otto). As shown in Table 1, the corresponding control monkeys in our series, gave the following results: 51% of 1000 cercariae (No. 34), 14% of 5000 cercariae (No. 35) and 29% of 5000 cercariae (No. 36).

The method of determining egg production differed in the two experiments. Vogel and Minning[5] used the Stoll-Hausheer dilution egg-counting technic and when it ceased to be positive they used a miracidial hatching concentration technic. To attain the same objective, we used a single procedure, the AMS III ether sedimentation, and counted all the eggs recovered from 2 grams of feces. The schedule of stool examination appeared to be about the same in both studies. Regardless of relative efficiency of the two procedures, similar egg-recovery patterns were obtained in both studies. In our study we recognized clearly the first of the three phases described by Vogel and Minning,[5] *i.e.*, the period of maximum egg production and subsequent decline. We also saw a prolonged period of low egg-output which seems to be comparable to their second and third phases. The latter phase covered that period when only the egg-hatching technic was positive, while the second phase included an earlier interval when the dilution egg-counting still gave positive results but low counts.

Our observations on egg production during the immunizing period also were similar to those of Vogel and Minning. Group I animals showed that *M. mulatta* is capable of suppressing egg production of a large number of worms within 200 days. In Group III a near-maximum egg production was reached after 200 days and the new worms which developed from the continued exposures merely sustained the numbers until about the 500th day, after which a sharp drop in eggs occurred. The interval of time that is required for the development of resistance in man may be quite different but surveys in endemic areas indicate that the pattern is similar.

Vogel and Minning[5] found that when resistant monkeys were challenged, the young worms (*S. japonicum*) did not reach the hepatic portal system, and histological studies led to the conclusion that most of them were stopped in the lungs. Our observations on *S. mansoni*, based on examinations made at various intervals after the final challenge, showed that a considerable number of worms did reach the hepatic portal system. However, it was evident that although a few of these worms may reach maturity in resistant animals, they are very much retarded in

their development, usually do not produce eggs, and are eliminated within 90 days or less after the exposure.

SUMMARY

Following a single exposure of *Macaca mulatta* to 1000 cercariae of *Schistosoma mansoni*, near-maximum egg recoveries persisted for only 60 to 90 days; they were at a low level by 200 days and negligible within 300 days. When challenged about 2 years after initial exposure, no increase or renewal of egg production occurred. When a series of 26 monthly exposures (25–50 cercariae each) was given, egg-counts increased for about 200 days, then remained at a moderately constant level for about 300 days, declined sharply thereafter, and became negligible before the series of exposures was completed. Another group of animals received varied combinations of moderate and light exposures at varied intervals. Some of these animals acquired only light infections and failed to resist additional exposures 250 or 500 days later. Among the animals used in the various experiments, there was evidence of considerable individual variation in the natural and acquired resistance.

The number of worms reaching the hepatic portal system of resistant animals after a second challenge was determined by examining the animals 6 to 65 days after exposure to cercariae. No worms were found in the liver 6 days after the challenge. At 15 days a few schistosomules were present. In animals examined at 22 to 42 days about 10% of the cercariae used in the challenge exposure had reached the liver. These worms were either undeveloped or stunted and apparently would not have reached maturity. At 55 and 65 days, stunted worms were present in two animals but in two others the worms had essentially disappeared. In one animal examined 97 days after the first challenge, no worms were found.

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# AN EPIDEMIC OF ST. LOUIS ENCEPHALITIS IN CAMERON COUNTY, TEXAS, IN 1957

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In late July and early August, 1957, a steadily increasing number of cases of encephalitis was reported from Cameron County, Texas. Since this county lies adjacent to Hidalgo, the scene of a large outbreak of St. Louis encephalitis (SLE) in 1954,[1-4] these reports were of unusual interest and early in August an intensive study of the outbreak was undertaken.

In all, 114‡ clinical cases were encountered during July and August, 99 of which occurred among civilians, 11 in members of the Armed Forces stationed at the Harlingen Air Force Base, and 4 in their dependents. The over-all attack rate was 155‡ per 100,000. Serological evidence from these cases and virus isolation from mosquitoes[5] confirmed the diagnosis of St. Louis encephalitis (SLE).

The basic purpose of this investigation was to document the relatively rare occurrence of epidemic SLE. In addition to the routine epidemiologic work-up, however, ancillary studies were made possible by the co-operative attitude of the community which, after the sensitizing effect of the 1954 SLE epidemic in Hidalgo County, was currently witnessing a similar occurrence in its midst. Efforts were directed at defining the actual incidence of infection throughout the community. Since the majority of infections were subclinical, this involved the serologic sampling of well individuals. The ratio of apparent to inapparent infections was studied in the population as a whole, with particular attention to variations in specific age groups. Data from these studies will be presented in this discussion. A report on the epidemic as it involved the military population has been published separately.[6]

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‡ In a previous publication concerning this epidemic[6] 120 clinical cases with an attack rate of 163 per 100,000 were reported. The corrected figures take into account revised diagnoses discovered subsequent to the first publication.

## METHODS

The epidemic was investigated jointly by the Texas State Department of Health and the Communicable Disease Center with the co-operation of the United States Fourth Army and the United States Air Force. Several State Sanitarians and Entomologists working toward definition and control of the mosquito population, had preceded the authors in entering the area.

The authors attempted to contact each case and secure data on age, sex, date of onset, and clinical course. To aid in this work, the records in the four hospitals in the area were made available. A case was accepted on the basis of final diagnosis by the attending physician.

Paired blood specimens were submitted by local physicians in 18 cases and in 5 others single specimens were available. Paired specimens were collected from all military case suspects and dependents. The blood samples were tested for complement fixation (CF) antibodies by the Laboratory of the Texas State Department of Health and the Laboratory of the U. S. Fourth Army, Fort Sam Houston, Texas. Specimens were run exclusively for SLE at the Laboratory of the U. S. Fourth Army with acetone extracted mouse brain antigen and 2 units of complement. The CF tests at the Laboratory of the Texas State Department of Health were performed with benzene extracted mouse brain antigen and 2 exact units of complement. Paired sera were tested against eastern and western equine encephalitis (EEE and WEE) and mumps. In both laboratories antigen was used at optimal dilution, this being the highest dilution of antigen giving highest serum titer.

Among the military population a random sample of 150 airmen who had not been clinically ill during the epidemic and had never received yellow fever vaccination were bled during the 6th week following the epidemic and the serum was tested for CF antibodies to determine the rate of inapparent infection. In these studies a

CF titer of +3 or +4 at 1:8 dilution was accepted as indicating recent SLE infection.[6] In the last week of the epidemic 367 civilians were bled in order to determine the inapparent infection rate for the resident population. Every attempt was made to collect blood from a representative sample of the population. About one-third of the specimens were collected from household contacts of cases. One-third were from screened hospital admissions, primarily those admitted to the surgical wards, and the remainder were drawn from a wide variety of sources including a prenatal clinic, a venereal disease clinic, blood donors, and a series of clubs and religious groups. Although these were not randomly chosen, it is felt that they are a fairly reliable representation of the various groups of the community. The age distribution of the sample differs widely from that of the population because of the greater relative accessibiiity of young adults. Again a CF titer reacting +3 or +4 at a 1:8 dilution was accepted as indicating SLE infection.

### METEOROLOGICAL AND ZOOLOGICAL BACKGROUND

Cameron County is a flat semi-tropical area at sea level, with mean temperatures in January of 60°F and in July of 84°F. Industrial agriculture is extensive and open ditch irrigation is common practice. In the year 1956 a total of 10.98 inches of rain fell, while between January and mid-June 1957 precipitation reached 20.72 inches. Late June, July, and August were completely dry. Standing water in ditches and arroyos was in evidence throughout the epidemic area. This pattern of heavy rain, then drought, then epidemic of SLE has occurred in other major outbreaks of the disease: St. Louis, 1933,[8] Hidalgo, 1954,[4] and Calvert City, Kentucky, 1955.[9] Standing water which has time to accumulate a high concentration of organic and inorganic salts apparently favors the breeding of the *Culex pipiens-quinquefasciatus* complex. This mosquito complex has been incriminated as the probable vector in most outbreaks of SLE east of the Rockies.[10] (In outbreaks in eastern Colorado, Kansas, and the high plains in Texas *C. tarsalis* has been the apparent vector.)

In April and May an increasing pest mosquito problem was controlled by the State and Armed Forces adultaciding and larvaciding measures. These practices were resumed and intensified in late July and early August during the epidemic.

Richard Eads, Entomologist, Texas State Department of Health, and his staff, collected adult mosquitoes and larvae in Cameron County and on the Air Force Base during the outbreak. Efforts were concentrated toward isolating pools of the extraordinarily abundant *Culex quinquefasciatus*; however, *Psorophora confinnis*, *Aedes vexans* and *sollicitans*, and *Anopheles quadrimaculatus* were also plentiful. A few larvae of *Culex tarsalis* were encountered but no adults were captured. A total of 34 pools, each containing approximately 50 unengorged mosquitoes, was collected and tested for virus activity. The SLE virus was isolated from 8 pools of *Culex quinquefasciatus* in the laboratory of Dr. J. V. Irons, Texas State Department of Health, by Wiseman *et al.*[5]

The animal reservoir for SLE was not studied. Cameron County has one of the highest wild bird populations in this country.[11] Species previously incriminated[12] as potential hosts for virus abounded. According to records of the Wild Life Refuge manager, white-wing doves reached an 8-year maximum; purple grackles, red-wing blackbirds and English sparrows were ubiquitous; and mourning doves and woodpeckers were plentiful. A factor influencing the unusual prevalence of wild birds in 1957 was the very extensive and early nesting in the citrus trees noted during the Spring. A frost in 1952 destroyed most of the citrus trees in the area and these trees did not re-establish themselves sufficiently to yield good crops until 1957.

Domestic fowl were infrequently noted in close proximity to known cases. Very few horses reside in the county and no equine encephalitis was reported, although both eastern and western equine encephalitis have been suspected in the area in past years.[13]

### RESULTS

*Clinical cases.* A total of 114 cases was reported from the western portion of Cameron County in an area with a population of 74,000 inhabitants. Blood specimens were collected from 42 individuals, 35 of whom showed evidence of CF antibody to SLE virus. Of these, 25 had a four-fold rise between acute and convalescent specimens. In five other cases where the initial titer was 1:16 or over, four-fold rise was not observed in the convalescent serum. In the remaining five cases in which CF antibody was demonstrated,

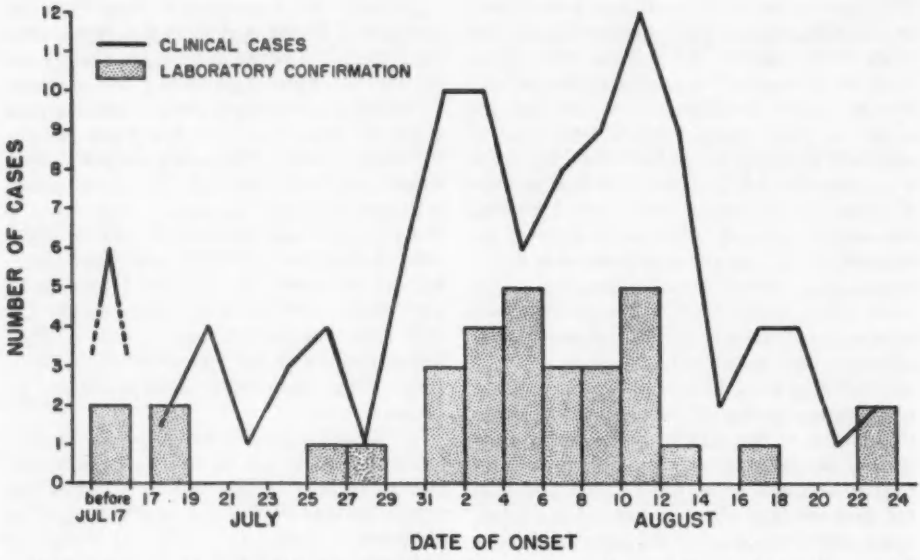


FIG. 1. Number of cases of St. Louis encephalitis in Cameron County, Texas, during the summer of 1957.

TABLE 1

*St. Louis encephalitis in Cameron County, Texas, in 1957*

| | Population | Reported cases | | | Clinical attack rates* |
|---|---|---|---|---|---|
| | | Male | Female | Total | |
| Civilian | 67,300 | 53 | 46 | 99 | 146 |
| Military personnel | 3,900 | 11 | — | 11 | 280 |
| Military dependents | 3,800 | 1 | 3 | 4 | 110 |
| Total | 74,000 | 65 | 49 | 114 | 155 |

* Rates per 100,000.

only one specimen of blood was available. One paired sera showed evidence of recent mumps infection while 6 were negative when tested for EEE, WEE, and mumps. In one instance where there was a diagnostic rise to SLE virus a titer of 1:16 to WEE was present in both acute and convalescent serum. On no other occasion was a titer to WEE greater than 1:4 encountered.

Clinically, the cases did not differ markedly from those reported in St. Louis[14] and Hidalgo.[2] The overwhelming majority of the clinically ill required hospitalization with more than half showing definite lethargy or mental changes. Very severe frontal headache and some degree of back and neck stiffness were almost universal. Three cases terminated fatally: a female, age 84, and two males, ages 60 and 86.

While 2 isolated cases occurred before July 1, the outbreak actually commenced in that month (Figure 1). Between July 29 and August 14, 72 cases occurred. The last reported cases occurred on August 23. The 35 cases in which the clinical picture was supported by laboratory evidence of SLE infection had onset dates similar to the general epidemic curve (bar graph portion of Figure 1), supporting the belief that the epidemic was predominantly SLE.

The epidemic area included the community of Harlingen with some 35,000 inhabitants and the Harlingen Air Force Base with 3,900 airmen and an equal number of dependents. San Benito, with a population of 18,000, was the only other urban center. Brownsville, located in Cameron County's southeastern corner, was not involved in the outbreak.

The overall attack rate (Table 1) was 155 per 100,000, while the attack rate among airmen was 280 per 100,000. Urban centers seemed to be involved somewhat earlier than were rural areas,

although no centrifugal patterns could be detected. The urban and rural attack rates were not appreciably different.

Of the 114 cases, 65 occurred in males and 49 in females. Considering civilians alone, 53 were among males and 46 among females (Table 1). Part of the excess of cases among males in the civilian population may result from the fact that a large number of braceros and migrant laborers were in the county during the epidemic.

The 114 infected individuals ranged in age from 14 months to 86 years (Table 2). The highest incidence appeared in young adults with almost half the cases occurring in individuals between 20 and 40 years of age. Most striking, however, was the fact that 24% of the cases appeared in people over 60, while according to the 1950 census[15] this age group accounted for only 7% of the population. Age specific attack rates could not be calculated because no data were available since 1950 and the population had increased 30% since that time.

*Inapparent cases.* Inapparent attack rates were analyzed separately for military and civilian populations. As stated previously,[6] there were 11 cases of clinical encephalitis among military personnel in a population of 3,900, giving a clinical attack rate of 280 per 100,000. To determine the inapparent attack rate, 150 airmen who were well throughout the epidemic were bled and CF antibodies were encountered in 27 instances. This gives an inapparent attack rate of 18% and a ratio of inapparent to apparent cases of 64:1.

In the civilian population of 67,300, there were 99 clinical cases, giving a clinical attack rate of 155 per 100,000. During the last week of the epidemic 367 individuals who had been well during the epidemic were bled. The sera of 111 of them contained CF antibodies for an inapparent attack rate of 30.5%. By comparing the inapparent to the apparent attack rates, it is seen that for each clinical case 209 subclinical infections occurred.

A breakdown of the inapparent infections in the civilian population is presented in Table 3. In the sample of 367 (208 males and 159 females), 56 males and 55 females were positive. This gives a somewhat higher rate of inapparent infections among females than among males, while more males suffered apparent infections than did females. These differences are not of sufficient magnitude to be statistically significant.

The age distribution of individuals with inapparent infections (Table 3) showed a remarkably even spread for 10-year age groupings. The overall percentage positive was 30.5. In the age groups between 10 and 70, where a minimum of 25 blood specimens for each 10-year span was analyzed, the positives fell between 20 and 37.8%. The small differences in age groups were not statistically significant ($p = 0.2$). This relatively even age distribution for inapparent infections differs markedly from the age distribution for clinically apparent illness where attack rates would appear to be considerably higher among older people.

TABLE 2

*Age and sex distribution of clinical cases of St. Louis encephalitis in Cameron County, Texas, during 1957*

| Age | Civilians | | | Military personnel | Military dependents | | | Total | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Male | Female | Total | | Male | Female | Total | Male | Female | Total |
| 0–9 | 2 | 6 | 8 | | 1 | 0 | 1 | 3 | 6 | 9 |
| 10–19 | 8 | 3 | 11 | 2 | | | | 10 | 3 | 13 |
| 20–29 | 12 | 7 | 19 | 7 | | 3 | 3 | 19 | 10 | 29 |
| 30–39 | 9 | 9 | 18 | 2 | | | | 11 | 9 | 20 |
| 40–49 | 5 | 2 | 7 | | | | | 5 | 2 | 7 |
| 50–59 | 4 | 4 | 8 | | | | | 4 | 4 | 8 |
| 60–69 | 2 | 7 | 9 | | | | | 2 | 7 | 9 |
| 70–79 | 5 | 4 | 9 | | | | | 5 | 4 | 9 |
| 80–89 | 3 | 0 | 3 | | | | | 3 | 0 | 3 |
| Unknown | 3 | 4 | 7 | | | | | 3 | 4 | 7 |
| Total | 53 | 46 | 99 | 11 | 1 | 3 | 4 | 65 | 49 | 114 |

TABLE 3

*Age and sex distribution of inapparent infections* of St. Louis encephalitis in Cameron County, Texas, during 1957*

| Age | Male | | Female | | Both sexes | | Per cent positive | | |
|---|---|---|---|---|---|---|---|---|---|
| | Pos. | Total | Pos. | Total | Pos. | Total | Male | Female | Total |
| 0–9 | 3 | 3 | 0 | 2 | 3 | 5 | Numbers insufficient | | |
| 10–19 | 9 | 22 | 5 | 15 | 14 | 37 | 40.9 | 33.2 | 37.8 |
| 20–29 | 13 | 41 | 20 | 51 | 33 | 92 | 31.7 | 39.2 | 35.9 |
| 30–39 | 10 | 56 | 7 | 29 | 17 | 85 | 17.9 | 24.1 | 20.0 |
| 40–49 | 6 | 38 | 12 | 33 | 18 | 71 | 15.8 | 36.4 | 25.4 |
| 50–59 | 8 | 28 | 8 | 18 | 16 | 46 | 28.6 | 44.4 | 34.8 |
| 60–69 | 5 | 16 | 3 | 9 | 8 | 25 | 31.3 | 33.3 | 32.0 |
| 70–79 | 2 | 4 | 0 | 2 | 2 | 6 | Numbers insufficient | | |
| Total | 56 | 208 | 55 | 159 | 111 | 367 | 26.9 | 34.6 | 30.2 |

* Complement fixing titer of 1:8 or more accepted as indicating SLE infection.

## DISCUSSION

During the summer of 1957 an epidemic of St. Louis encephalitis occurred in Cameron County, Texas. A total of 114 cases was reported, of which 35 received some laboratory confirmation. The documentation of this epidemic has provided an opportunity to compare the epidemiologic patterns with those of previous epidemics of SLE and particularly of the outbreak which occurred in adjacent Hidalgo County in 1954. Of considerable additional interest were the results of serological surveys to determine inapparent attack rates. A marked disparity between the age distribution of apparent and inapparent infections was demonstrated.

In recent years cases of encephalitis have been reported from Cameron County each summer, but no defined epidemics have occurred. During the epidemic in Hidalgo County in 1954 only sporadic cases were observed in Cameron County. Since these counties are adjacent and present identical environments, it is difficult to understand why the epidemic was confined to one county in 1954, when the other proved itself three years later to be quite capable of sustaining a similar outbreak. Equally difficult to explain is the fact that the 1957 epidemic reached its peak in early August, 3 weeks earlier than the 1954 peak, although ecologic and climatic conditions did not differ appreciably. Both epidemics followed a cycle of heavy rain, then long drought during which standing water provided breeding areas for vast numbers of *Culex quinquefasciatus* mosquitoes from which SLE virus was isolated. The bird reservoirs were also similarly large.

During the epidemic in Cameron County 114 cases were reported, 99 of which occurred among civilians, while 11 cases were in airmen attached to the Harlingen Air Force Base and 4 were in their dependents. The attack rate for the entire community was 155 per 100,000. The 11 cases in the military population gave an attack rate of 280 per 100,000. In the two counties involved in the St. Louis epidemic in 1933 (575 and 520 clinical cases, respectively) rates were 69 and 212 per 100,000. In the Hidalgo outbreak of 373 cases the attack rate was 163 per 100,000. The definition of the denominator population at risk determines the attack rate and for this reason the specific figures in these epidemics are not entirely comparable. As can be seen, however, the order of magnitude of attack rates has not differed widely. In Cameron County, no great variation was noted between the rates in urban and rural areas, and actual spread of infection from a center was not demonstrated.

The incidence of cases was slightly higher in males than in females (65 to 49) but the difference is less impressive when cases among military personnel are deleted. In the St. Louis epidemic the cases were divided equally between sexes. In Hidalgo the rates among females were slightly higher. In the outbreaks in the East Central States in 1955 and 1956[10] cases among females

TABLE 4

*Ratio of clinical illness to inapparent St. Louis encephalitis infection*

| Epidemic | Ratio |
|---|---|
| Cameron County, Texas—1957 | |
| Civilian | 209:1 |
| Military | 64:1 |
| Hidalgo County, Texas—1954 | 500:1 |
| Calvert City, Ky.—1955 | 16:1 |
| Louisville, Ky.—1956 | 200:1 |

were in considerable excess, while in the Western States the numbers were equal.

Almost half of the cases occurred in individuals between 20 and 40 years of age. Only 8% appeared in children under age 10. In the group over 60, comprising less than 7% of the population, 24% of the cases occurred, implying considerably higher attack rates among elderly people. This age distribution follows very closely that of almost all epidemics of SLE east of the Rockies, and is in contrast to the age distribution for both eastern equine encephalitis (EEE) and western equine encephalitis (WEE). In both EEE and WEE the majority of cases occur in infants and young children. For reasons poorly understood, cases of SLE occurring in the Mountain and Pacific areas follow more closely the age pattern of WEE except for the absence of SLE in the first year of life.[10, 16]

Clinically, the Cameron County cases were not remarkable. Three deaths occurred, giving a case fatality percentage of 2.6. In Hidalgo the case fatality percentage was 2.7 of the known cases. In St. Louis in 1933 the case fatalities were about 20% and rates in 1955 and 1956 in Kentucky were similarly high, with 19 and 13%, respectively.[10, 17] Deaths in all these outbreaks, including those in Cameron County, occurred almost exclusively in elderly people.

The problem of determining the ratio of inapparent infections to apparent infections is a very difficult one. Clinical illness in most of the arthropod-borne encephalitidies, is an iceberg phenomenon representing a small fraction of the total infections in a community. The actual ratio, however, will vary with the ability to find clinical cases, the criteria used in accepting clinical cases, the definition of the denominator population at risk, the selection of the control groups, the tests employed in determining inapparent infection, and the variety of natural factors involved. It is not surprising, therefore, that a wide range of ratios of inapparent to apparent cases exists in the literature (Table 4). In Calvert City, Kentucky, 1955,[9] the ratio was 16:1, while in Louisville, Kentucky, 1956,[10] it was 200:1, and in Hidalgo County, Texas, 1954,[1] 500:1. Ratios from 500:1 to 1000:1 have been described for Japanese B encephalitis.[18] In the present study the ratio among civilians was 209:1 and among military personnel 64:1.

The differences between the military and civilian populations during the same epidemic tend to suggest a higher degree of immunity among the resident population. The airmen, however, represent a highly selected group comprised entirely of young adult males with ready access to excellent and free medical care. With the only measuring tool being the CF test, interpretation of the data must be guarded.

As has been pointed out, the clinical attack rates for SLE are consistently higher among older individuals. Lumsden,[19] who deduced the mosquito transmission of SLE in the epidemic in St. Louis in 1933, postulated that the higher attack rate in the aged was secondary to a higher infection rate in this group. He reasoned that the habits of older people regarding recreation, hygiene, and dress made them more readily accessible to the bites of vector mosquitoes. Ranzenhofer *et al.*,[9] in Calvert City, Kentucky, 1955, presented evidence supporting the concept that higher clinical attack rates resulted from higher infection rates in the older age groups. In their study of 13 clinical cases, they analyzed 98 control blood specimens for evidence of inapparent infection. Older age groups showed progressively higher attack rates for both clinical and inapparent infections.

In the present study contrary information has come to light. While the attack rate in the 99 clinical cases was considerably higher in the older age groups there was no significant variation by age among the 367 control specimens analysed.

These data indicate that the infection rate among the aged was no higher than for the rest of the community. Higher clinical attack rates in older people appear to be related to the host response to the infection rather than to a higher

rate of infection. The evidence suggests that a given infection with SLE virus is more likely to produce clinical symptoms in an older individual than in a younger person.

SUMMARY

An epidemic of St. Louis encephalitis occurred during July and August of 1957 in Cameron County, Texas. A total of 114 clinical cases was reported, of which laboratory evidence of SLE infection was available in 35 instances.

Ecologic conditions were conducive for SLE since this area has a very large wild bird population, and after heavy rains followed by drought *Culex quinquefasciatus* mosquitoes were extraordinarily abundant. SLE virus was isolated on 8 occasions from these mosquitoes.

The attack rate for the entire community was 155 per 100,000. The urban and rural rates were similar and no spread from foci was noted.

The age distribution of cases was similar to that in previous SLE epidemics east of the Rockies, with greatest numbers occurring in young adults and highest attack rates in older age groups. Cases among males were somewhat more prevalent than those among females. Three deaths occurred, all in people over 60.

The clinical attack rates were higher in a military population within the epidemic area than among the resident population. Inapparent attack rates, however, were higher among civilians (30% to 18%). The ratio of inapparent to apparent infections in civilians was 209:1, while in the military group it was 64:1.

The inapparent infection rate was the same in all age groups, while clinical attack rates were higher in the older age groups, suggesting a greater susceptibility to symptomatic illness among older people.

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# THE POSSIBLE EFFECTS OF pH AND SPECIFIC GRAVITY ON THE ETHER-SEDIMENTATION PROCEDURE IN CONCENTRATING EGGS AND CYSTS

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Empirical modifications of the original Telemann ether-sedimentation procedure[1] for concentration of intestinal protozoa and helminth eggs are sufficiently used to warrant investigation of basic factors which influence their action. Many minor alterations had been imposed on the formalin-ether modification of this technic[2] by its author without significantly altering its efficiency. This seemingly constituted a merit, making this technic easily adaptable to conditions of different laboratories and minimizing the effect of changes imposed by different technicians. Alterations of the pH and specific gravity of the sedimentation medium gave the first indication that basic factors had been found.[3]

The choice of the formalin-ether procedure for the current study can be justified on the basis of simplicity, adaptability to the experimental variables, and the fact that its merits and efficiencies are well established.[4-10] Furthermore, it is doubtful if certain other of the ether-sedimentation procedures could have been adapted to this experiment. Some utilize highly acid solutions, well beyond the range of pH values used here, *e.g.*, the AMS III technic.[11] High acidity is a basic feature and alteration would actually constitute a new technic. The "MIFC" method[6] is distinctive because it employs the Merthiolate-iodine-formalin mixture of Sapero and Lawless[12] as the concentration medium. This mixture is a complex of many substances, which made it experimentally unsuitable; *e.g.*, the Merthiolate contains alcohol, which was the agent used in the present study to alter specific gravity. Eliminating the alcohol would not have been feasible, and replacing the MIF complex with water by repeated washings would surely have eliminated its unique features, the result being formalin fixed feces. Adapting the formalin-ether technic was merely a matter of replacing the formalin with buffered aqueous solution of varied pH values, to which alcohol was added as a second variable.

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## METHODS

Selected stools containing various eggs and cysts were concentrated in parallel series, using buffered aqueous solutions with pH values of 4, 7 and 10. McIlvaine's buffer system, combining 0.1 M citric acid and 0.2 M disodium phosphate, was used for obtaining solutions of pH 4 and 7; and for pH 10, the Clark-Lubs (traditional) system, combining 0.2 M KCl and 0.2 M $H_3BO_3$ with 0.2 M NaOH. A second series of solutions, commercially prepared by Fisher Scientific Company (Standard Buffer Solutions of pH 4, 7 and 10), was used on a limited number of specimens for comparison; results were similar and data were combined.

Fecal suspensions were prepared from individual stools in 7.5% formalin. This concentration proved as effective as the 10% solution originally recommended.[2] The formalinized specimens were sometimes kept for several weeks, but tests proved that loss of technic efficiency is minimal and not apparent within 1 month; furthermore any decline would have been equally imposed on all test categories. The "stock" specimens were strained through two layers of gauze and each was roughly diluted so that a 10 ml aliquot yielded a quantity of centrifuged feces within the range of 0.5 to 1.5 ml; tests had shown that the technic tolerates this degree of variation. Replicate aliquots from individual specimens did not vary from one another. After thorough mixing, 10 ml portions of each specimen were pipetted into a series of three 15 ml centrifuge tubes. Subsequent handling of each series of tubes was essentially identical, except for the experimental variables. The specimens were centrifuged, the

formalin supernate was decanted, and 10 ml of each buffered solution was added to the respective tubes. Three ml of ether was added to each tube, which was then thumb-stoppered and shaken vigorously (30 times). Following centrifugation, the superficial debris and supernate were decanted, the tubes were wiped with cotton swabs and microscopic preparations were made according to the original description of the procedure.[2]

Technic effectiveness was determined by both diagnostic efficiency, and semiquantitative egg- and cyst-counts. Since the latter is difficult to standardize and determination of diagnostic efficiency requires relatively large numbers of cases, the use of both criteria was deemed desirable. The presence or absence of cysts and eggs was based on a complete examination of the fecal concentrate that could be accommodated at optimal density under a 22 x 22 mm coverslip. The cyst- and egg-counts were based on two representative l.p. microscopic lanes across the coverslip (about one-sixth of the preparation), except for *S. japonicum*, for which all eggs on each slide were counted. To count all other eggs was judged to be an ill-advised burden. Standardization of smears for cyst- and egg-counts was not ideally accomplished. It would have been simple to impose the "dilution" procedure, but this was judged a poor criterion for ether-sedimentation fecal concentrates, because of the superimposed variability in the amount of debris recovered. It appears that the basis of standardization should be the number of eggs and cysts in that portion of the debris that can be accommodated at optimum density in one slide preparation. This criterion was imposed through the judgment of the technicians, whose extensive experience was assurance of a high degree of uniformity for this subjective handling of the problem. It now appears probable that the amount of fecal sediment to be used in such slide preparation could be standardized by adapting the procedure of Beaver[13] for obtaining uniformity of smear density.

For an initial series of stools, egg- and cyst-counts and diagnostic efficiencies were both employed, while for a second larger series, only diagnostic efficiency was used. Individual technicians examined all three pH-varied samples for a single stool when counts were made, but when only diagnostic efficiencies were being determined, they merely examined equal numbers of specimens for each experimental category without reference to previous results. A series of plus-count categories was used to quantitate cyst recovery, from which a comparative index figure was determined after the manner of Hunter,[14] as adapted by Tigertt *et al.*[15] (for method see Table 1).

The possible effect of reducing specific gravity was observed by adding alcohol to solutions of varied pH values in amounts giving a final concentration of 20%.

## RESULTS

*Effect of pH on the efficiency of the ether-sedimentation procedure.* For 50 stool specimens with fertile *Ascaris* eggs, the mean counts for pH values of 4, 7 and 10 were *7*, *20* and *60* eggs, respectively, or a ratio of approximately 1:3:9 (Table 2). Analysis of results by the ranking test of Wilcoxon, as given by Bliss and Calhoun,[16] indicated that the differences were significant. Diagnostic efficiency was also affected, as only 84% of the infections were diagnosed with pH 4, while at both pH 7 and 10, 98% were identified. For unfertile *Ascaris* eggs the results were somewhat different, but the alkaline pH was the best.

Hookworm results were the inverse of the above, mean egg-counts for 38 cases being *25*, *25* and *1* for pH 4, 7 and 10. Also the ineffectiveness of pH 10 was apparent diagnostically, with only 41% of the infections being identified, as compared with 98 and 94% for pH 4 and 7.

*Trichuris* egg recovery was still different, being less influenced by pH, and diagnostic efficiencies were unaffected.

A second series of 229 specimens was used for additional evaluation of diagnostic efficiencies for the varied pH values. It is apparent (Table 2) that the results were consistent with those of the first series: for *Ascaris* eggs, results were inferior at pH 4 and equally superior at pH 7 and 10; the reverse was true for hookworm and *Trichostrongylus sp.* eggs, with low efficiency at pH 10, while diagnosis of *Trichuris* was unaffected.

In all, 82 infections by *S. japonicum* were identified among 330 stool specimens utilized, and for these positive cases, diagnostic efficiencies with pH values 4, 7 and 10 were 20, 55 and 78% respectively. Correspondingly, mean egg recoveries, on single-slide preparations of all positives, were 1, 5 and 10 (Table 2).

For intestinal protozoa (Table 3), only minimal

TABLE 1

*Method of estimating and comparing protozoan cyst recoveries*

| Category symbols | Cyst count* | | Index figure(one-half, max) | No. cases | Product I.F. × cases |
|---|---|---|---|---|---|
| | Range | Maximums | | | |
| 0 | 0 | 0 | 0 × 7 = | | 0 |
| + | 1–50/slide | 50 | 25 × 17 = | | 425 |
| 2+ | 1–50/h.p. lane | 50[2] | 1,250 × 12 = | | 15,000 |
| 3+ | 1–50/h.p. field | 50[2] | 62,500 × 10 = | | 625,000 |
| | | | Totals | 46 | 640,425 |
| | | | Mean | = | 13,922 |
| | | | Cyst index | = 13.9 | |

* Since 22 mm coverslips approximate 50 and 50[2] h.p. micro-lanes and fields, respectively, the choice of 50 cysts was indicated as a range maximum for the lowest positive category.

TABLE 2

*Helminth egg recoveries and diagnostic efficiencies obtained with varied pH values*

| | *Ascaris* fertile (50)* | *Ascaris* unfertile (15) | Whipworm (90) | Hookworm (51) | *Trichostrongylus* (11) | *S. japonicum* (24) |
|---|---|---|---|---|---|---|
| Series 1†—Mean egg count per specimen—two l.p. micro-lanes | | | | | | |
| pH 4 | 7 | 1 | 32 | 25 | 1 | 1‡ |
| pH 7 | 20 | 4 | 41 | 25 | 1 | 5‡ |
| pH 10 | 60 | 5 | 20 | 1 | 1 | 10‡ |
| Series 1—Diagnostic Efficiency—Per cent of infections identified | | | | | | |
| pH 4 | 84 | 60 | 97 | 98 | 69 | 8 |
| pH 7 | 98 | 47 | 97 | 94 | 54 | 46 |
| pH 10 | 98 | 93 | 98 | 41 | 54 | 79 |
| Series 2—Diagnostic efficiency—per cent of infections identified | | | | | | |
| | (73) | (43) | (211) | (81) | (37) | (58) |
| pH 4 | 77 | 56 | 96 | 95 | 89 | 24 |
| pH 7 | 90 | 86 | 98 | 95 | 38 | 59 |
| pH 10 | 95 | 88 | 96 | 33 | 46 | 78 |

* Figures in parenthesis indicate number of infections.

† Series 1 and 2 included 101 and 229 stools respectively.

‡ Based on all 82 infections in Series 1 and 2.

differences in diagnostic efficiencies were noted for the several pH values employed; however, the differences were consistently favorable to pH 7 for all species. Quantitation of cysts revealed that

TABLE 3

*Influence of pH on the diagnosis of intestinal protozoa*

| | No. of infections | Diagnostic efficiency (per cent identified) | | | Cyst recoveries (index numbers*) | | |
|---|---|---|---|---|---|---|---|
| | | pH 4 | pH 7 | pH 10 | pH 4 | pH 7 | pH 10 |
| *E. histolytica* | 46 | 85 | 91 | 87 | 14 | 25 | 11 |
| *E. coli* | 127 | 93 | 99 | 88 | 21 | 24 | 14 |
| *E. nana* | 74 | 96 | 99 | 76 | 29 | 32 | 12 |
| *G. lamblia* | 36 | 94 | 94 | 83 | 42 | 40 | 25 |

* See "Methods"—numbers represent thousands of cysts, the mean for all cases of a category.

numbers were highest for pH 7, only a little lower at pH 4, with the exception of *E. histolytica*, while at pH 10 numbers were reduced almost 50%.

*The effect of lowered specific gravity on the efficiency of the ether-sedimentation procedure* (*Table 4*). The use of the Merthiolate-iodine-formalin solution[12] as a medium for the ether-sedimentation procedure, MIFC procedure of Blagg *et al.*,[6] involves a reduction of specific gravity in the system. This undoubtedly is due to the alcohol and acetone in the tincture of Merthiolate. This influence of reduced specific gravity has been tested by including alcohol in each pH solution to the extent of 20%, final dilution. By so doing, egg recoveries were changed.

The results for the categories "without alcohol" were generally similar to those of previous series where pH alone was being evaluated. With the introduction of alcohol, remarkable increases in *Ascaris* eggs occurred, but the largest number of

TABLE 4

*Effect of adding alcohol to the varied pH solutions as a means of reducing the specific gravity*

| Helminthic infections | No. of infections | pH 4 | | pH 7 | | pH 10 | |
|---|---|---|---|---|---|---|---|
| | | Without alcohol | With alcohol | Without alcohol | With alcohol | Without alcohol | With alcohol |
| Mean egg counts per specimen—two l.p. micro-lanes | | | | | | | |
| *Ascaris* (F) | 20 | 5 | 104 | 15 | 202 | 68 | 160 |
| Whipworm | 50 | 22 | 58 | 29 | 43 | 17 | 7 |
| Hookworm | 27 | 17 | 17 | 13 | 8 | 1 | 0.3 |
| *S. japonicum** | 26 | .04 | 3 | 0.3 | 10 | 1.8 | 2.7 |
| Diagnostic efficiency—per cent of infections identified | | | | | | | |
| *Ascaris* (F) | 20 | 75 | 90 | 90 | 100 | 95 | 100 |
| Whipworm | 50 | 96 | 98 | 96 | 98 | 88 | 72 |
| Hookworm | 27 | 89 | 89 | 89 | 78 | 41 | 11 |
| *S. japonicum* | 26 | 4 | 54 | 12 | 100 | 54 | 54 |

* All *S. japonicum* eggs per slide were counted.
(F) Fertile eggs present.

eggs was obtained at pH 7 rather than at pH 10. There was also an increase in *Trichuris* eggs at pH 4 and 7, with a relative decline at pH 10. Recovery of hookworm eggs was essentially uninfluenced by the use of alcohol. For *S. japonicum* the alcohol gave a marked increase in eggs and as for *Ascaris* the greatest efficiency was linked with pH 7. Marked gains in diagnostic efficiency were not apparent, except for *S. japonicum* at pH 7; however for *Ascaris*, whipworm and hookworm, efficiency was of a high order, even without alcohol.

Detection of protozoan infections was not increased by the use of alcohol; in fact there was the suggestion of a decrease, but the series was too small to judge.

## DISCUSSION

The data accumulated indicate that pH influences the performance of the ether-sedimentation procedure, particularly the recovery of certain helminth eggs. A question might be raised as to whether the chemicals per se used in preparing the buffered solutions affected the results. A method of clarifying this point has not been devised; however, the solutions were diluted as much as one part to three of water without affecting the results. Likewise, the alcohol itself may have been an influence, as well as the lowered specific gravity which it imposed. This was suggested by a variation in the amounts of fecal sediment recovered. Without alcohol, the quantity of fecal sediment increased progressively with increase in pH value, whereas it was scant with alcohol at pH 10. This appears contradictory to the expected influence of specific gravity alone. Appropriate chemicals for lowering specific gravity are limited. Acetone (4%) as well as alcohol (20%) is a constituent of tincture of Merthiolate. On the basis of a limited comparison, no added benefit was noted by including acetone with the alcohol. Other chemicals considered were not miscible with water.

Temperature has been suggested as another possible factor in the function of the ether-sedimentation procedure, but it was not investigated. Wetting agents have been used effectively with ether-sedimentation and a triton was recommended for the formalin-ether technic by Maldonado.[8] Unfortunately a peculiar reaction occurs between iodine and many wetting agents, which precludes their use, if iodine is desired for staining cysts. A satisfactory wetting agent which does not react with iodine has not yet been found. A number of the common fixatives have been extensively tested in parallel with formalin, but no added benefits were derived.

The Merthiolate-iodine-formalin mixture of Sapero and Lawless,[12] used by Blagg *et al.*[6] as the medium for ether-sedimentation, appears to embrace a complex of factors. The fixative is formalin, with iodine and the coloring matter of

the Merthiolate (probably eosin) serving as stains. The possible influence of the several constituents of Merthiolate tincture has been studied. Eosin alone has not been found to have any effect on the technic. A Merthiolate solution of the same concentration as that of the tincture, when applied singly, imposed a highly alkaline pH, but this is nullified in the MIF by the iodine, giving the mixture an acid reaction. Alcohol occurs in tincture of Merthiolate to the extent of 20% and acetone 4%; these of course reduce the specific gravity of the mixture. Further, a wetting agent in great dilution is also present. When iodine is omitted from MIF, the results are little affected, except for hookworm, for which recovery is greatly reduced. The MIF mixture is a striking complex, which afforded a suitable medium for ether-sedimentation.

It is thought that the ether-sedimentation procedure may involve a complex of physico-chemical factors which may enhance or reduce efficiency when combined in varied relationships. Such a complexity might account for seemingly contradictory findings, and one such is already apparent: Increased effectiveness of schistosome egg recovery with an alkaline medium, as observed in this study, is in contrast to the efficiency of certain highly acid media which were developed specifically for this type of egg. It is believed that compensatory factors may be involved, *e.g.*, the use of a detergent.

As originally described, the formalin-ether technic[2] used formalin (10%) as both fixative and the medium for concentration. At this dilution, the formalin of two manufacturers had pH values of about 4, which approximates the acid pH used in the current investigation. This degree of acidity proved very unfavorable for *S. japonicum* eggs, and to a lesser extent for *Ascaris* eggs. The choice of a concentration medium for the formalin-ether technic could be varied according to diagnostic objectives, but for inclusive routine diagnoses it appears that a near-neutral solution with alcohol (20% final dilution) is favorable. This can be accomplished either by replacing formalin with the buffered alcoholic solution, as was done in this study, or by using a buffered formalin-alcoholic mixture as the fixative and retaining it as the medium of concentration.§ The latter procedure has been tried with seemingly favorable results, but further evaluation is indicated. Trophozoites have been recovered by using the alcoholic medium, but this merit has not been adequately evaluated.

§ A buffered formalin-alcohol solution (near pH 7) for further testing may be prepared as follows: dissolve 17.6 grams of citric acid and 88 grams of sodium phosphate, dibasic, in 2000 ml of water, dilute to 3600 ml, and add 400 ml of formaldehyde solution (37%) and 1050 ml of alcohol (95%). Because of the effect on specific gravity, the possibility of reducing buffering agents by one-half might be considered.


## SUMMARY

The effect of varied pH values (4, 7 and 10) on the diagnostic efficiency of the ether-sedimentation procedure has been investigated. Fertile *Ascaris* and *Schistosoma japonicum* eggs were recovered in greatest numbers with a pH value of 10, and were sufficiently reduced in number at pH 4 to adversely affect the diagnostic efficiency. Conversely, hookworm eggs were best recovered at pH 4 and 7, with almost complete loss at pH 10. Although whipworm eggs were most effectively recovered at pH 7, diagnostic efficiency was not appreciably affected by the pH variable.

An attempt was also made to determine the effect of lowered specific gravity. Alcohol, to the extent of 20%, was introduced into each pH solution. The nature of the results suggested that the alcohol per se was an influence, but in all test solutions the numbers of *Ascaris*, whipworm and *S. japonicum* eggs were greatly increased, particularly at pH 7, while hookworm was little affected.


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# ISOLATION OF PHLEBOTOMUS FEVER VIRUS FROM *PHLEBOTOMUS PAPATASI**

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The hairy midge or sandfly, *Phlebotomus papatasi*, has long been regarded as the probable vector of phlebotomus (sandfly) fever. Evidence incriminating the sandfly includes experimental human to human transmission,[1] similarities in the geographic and seasonal distribution of *Phlebotomus* flies and the disease,[2] and a marked reduction in the incidence of the disease following control measures directed against *P. papatasi*.[1, 3, 4] Although the virus of phlebotomus fever has been isolated repeatedly from man[5, 6] and has been extensively studied in the laboratory for the past decade, its recovery from wild-caught, naturally-infected sandflies has not been reported. Data on the isolation of the virus from wild-caught *P. papatasi*, collected from human dwellings in suburban Cairo, Egypt, are presented herein.

## MATERIALS AND METHODS

*Phlebotomus collections and identification.* The area within a 20 km radius of metropolitan Cairo, known from earlier work to be endemic for phlebotomus fever,[5, 6] was chosen as the study area. Beginning in late April 1959, human dwellings in villages located within the area were surveyed for the presence of *Phlebotomus* flies. Although sandflies were frequently seen, particularly in bedrooms, they were not found in a sufficiently large number in any one area to warrant their collection for isolation studies. Many factors, such as the small size of the fly, inadequate lighting, lack of contrast between the buff color of the fly and the mud walls of the local houses, and the thatched or reeded ceilings, made detection of the sandflies difficult. In the ensuing 7 weeks, only 2 of 16 localities surveyed, Barada and Qaranfil villages in Qalyubia province, showed promise as potential collection sites. Toward the middle of June, a third site was found which provided the major part of the insects used in the study. Designated El Amiriya, it consists of a 45 building apartment house development located on the northern city limit of Cairo and provides low-cost housing for families resettled from slum areas. In addition to providing large numbers of sandflies for isolation purposes, the development was epidemiologically advantageous as its high infant population afforded an excellent source of phlebotomus fever virus.

The insects, which were usually found resting in the corners of bedrooms near the ceiling, were collected in aspirator tubes by a team of 2 to 4 persons. Collections were made between 9:00 a.m. and 1:00 p.m., and the catch was returned to the laboratory for identification and isolation processing. Following division by sex, female *Phlebotomus* were identified to species according to the key of Kirk and Lewis,[7] pharygeal structure being used as the identification criterion. Heads of females were dissected from the bodies and mounted on numbered slides for identification; bodies were placed in similarly numbered holes of lucite spot plates and held at 4°C for 1 to 3 hours until species identification was completed. Bodies of phlebotomine flies which were not identified as *P. papatasi* were removed and the remaining *P. papatasi* bodies were pooled for virus isolation attempts. Because of the time-consuming nature of the identification procedures, only about 35% of the total female catch was identified to species; the remainder of the insects were pooled without identification. Approximately 80% of the females collected were engorged; because of the relatively small proportion of non-engorged females the two groups were not processed separately.

*Virus isolation and identification.* Because of the low virulence of phlebotomus fever virus for the suckling mouse and the probability that the sandflies would contain only a minimal amount of virus, every effort was made to avoid unnecessary dilution of the sandfly suspensions.

* The opinions and assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Navy Department or the naval services at large.

Based on the weight of 100 *P. papatasi* bodies (23 ± 3 mg), a 10% suspension of each pool was prepared using 0.2 ml of diluent for every hundred sandflies. The sandflies were ground in a small porcelain mortar in a diluent consisting of 50% normal rabbit serum in 0.02 M phosphate-buffered saline (pH 7.6). After centrifugation of the suspension at 8,000 rpm for 10 minutes (RCF = 2,800 G) in a Misco microcentrifuge, the supernate was withdrawn. Half the supernate was added to an equal volume of antibiotic solution, containing 2,000 units of penicillin and 2,000 μg of streptomycin sulphate per ml, and allowed to stand at room temperature (21°C to 25°C) for 45 minutes; the remaining supernate was stored without antibiotic in a sealed glass ampule at −70°C for reisolation attempts. After being cultured, the sandfly suspension was inoculated without further dilution into 2 litters of 1 to 2 day old suckling mice. Mice received 0.01 ml of inoculum intracerebrally and were observed for signs of illness for 3 weeks after inoculation. The brains were harvested from mice becoming ill or dying during this period and, when bacteriologically sterile, were further passed in normal mice. When it became apparent that a virus had been isolated, the agent was passed until it consistently caused the death of mice inoculated with a $10^{-4}$ dilution, at which time complement-fixing antigens were prepared.

Viral isolates were identified by complement-fixation using specific hyperimmune antisera against a variety of arthropod-borne viruses. Complement-fixing antigens consisted of high speed (10,000 rpm for 1 hour) supernates of 20% infected suckling mouse brain suspensions in unbuffered saline. Hyperimmune sera were prepared by immunizing mice with two weekly intraperitoneal injections of formalinized 10% infected mouse brain suspension followed by two weekly intraperitoneal injections of live virus. The animals were bled on the 10th day after the last injection. When separated, the sera were lyophilized and stored at −20°C.

Attempts were made to reisolate virus from original sandfly suspensions by inoculating suckling mice with material which had been stored at −70°C for 2 to 4 months.

As a precaution against contamination of unknown isolation material, all work with known viruses was performed in a room separate from the one used for virus isolation procedures. Similarly, animals inoculated with unknown preparations were kept in a separate room.

## RESULTS

*Sandfly collections and identification.* From 12 June to 10 November 1959, 33,100 *Phlebotomus* flies were collected from apartment dwellings in El Amiriya in suburban Cairo; of these, 26,730 were females. Of 8,727 females identified to species, less than 1% could not be identified as *P. papatasi*, usually because of technical difficulties in mounting. Thus, *P. papatasi* appeared to be the only phlebotomine species entering human dwellings in this area. Table 1 summarizes the semi-monthly catches for the entire collection period. Since collection methods were relatively constant, a valid comparison can be made between average semi-monthly catches; such comparison reveals a peak in the sandfly population during August. Owing to the precipitous decline in the sandfly population after the middle of October it was not considered worthwhile to continue collections for isolation purposes.

*Virus isolations and identification.* Viruses were isolated from one pool of *P. papatasi* collected on 19 August and from three pools of unidentified *Phlebotomus* (presumably *P. papatasi*) collected on 20 August, 24 August, and 13 October, respectively (see Table 2). In all instances, from 1 to 3 mice became ill 16 to 19 days after inoculation with sandfly suspensions. It is of interest that on subsequent passage the three August isolates adapted to mice much more readily than did the October isolate. By the fourth suckling mouse passage, the early isolates consistently killed mice between the 6th and 10th post-inoculation days and titered between $10^{-5.5}$ and $10^{-6.5}$. The October isolate, on the other hand, was much less virulent for suckling mice; by the eighth passage, it produced mortality only after 9 to 12 days and titered no higher than $10^{-4.5}$.

Virus was reisolated from all 4 of the sandfly suspensions which originally yielded virus.

The results of complement-fixation tests, in which antigens prepared from the 4 isolates (AR 43-59, AR 44-59, AR 46-59 and AR 60-59) were tested against sera hyperimmune to a variety of arthropod-borne viruses, are presented in Table 3. The data clearly indicate that all 4 virus isolates are immunologically related to or identical with the Sicilian type of phlebotomus fever virus. No reactivity was observed between

TABLE 1

*Summary of semi-monthly* Phlebotomus *collections from El Amiriya in suburban Cairo*

| Collection period | No. of *Phlebotomus* collected | | | Collection days | Average catch per collection day |
|---|---|---|---|---|---|
| | Females | Males | Total | | |
| 1–15 June | 80 | 15 | 95 | 1 | 95 |
| 16–30 June | 1,618 | 549 | 2,167 | 5 | 433 |
| 1–15 July | 2,476 | 791 | 3,267 | 4 | 817 |
| 16–31 July | 3,998 | 975 | 4,973 | 6 | 829 |
| 1–15 August | 4,709 | 1,029 | 5,738 | 4 | 1,434 |
| 16–31 August | 6,671 | 1,588 | 8,259 | 6 | 1,376 |
| 1–15 September | 2,620 | 563 | 3,183 | 3 | 1,061 |
| 16–30 September | 2,072 | 515 | 2,587 | 4 | 647 |
| 1–15 October | 2,132 | 300 | 2,432 | 4 | 608 |
| 16–31 October | 257 | 32 | 289 | 1 | 289 |
| 1–15 November | 97 | 13 | 110 | 1 | 110 |
| Total | 26,730 | 6,370 | 33,100 | 39 | 849 |

TABLE 2

*Summary of* Phlebotomus *pools processed for virus isolation*

| Month | Number of pools tested | | Average number of sandflies/pool | Phlebotomus fever virus isolations |
|---|---|---|---|---|
| | *P. papatasi* | Unidentified *Phlebotomus* | | |
| June | 10 | 0 | 141 | 0 |
| July | 20 | 0 | 243 | 0 |
| August | 6 | 13 | 616 | 1 (from pool of 649 *P. papatasi*)<br>2 (from pools of 805 & 689 unidentified *Phlebotomus*) |
| September | 0 | 7 | 607 | 0 |
| October | 0 | 3 | 655 | 1 (from pool of 657 unidentified *Phlebotomus*) |
| Total | 36 | 23 | 410 | 4 |

TABLE 3

*Identification of phlebotomus fever virus isolates by complement-fixation*

| Hyperimmune sera* | CF antigens | | | | | |
|---|---|---|---|---|---|---|
| | AR43-59 | AR44-59 | AR46-59 | AR60-59 | Phleb. fever (Sicilian) | Phleb. fever (Naples) |
| Phlebotomus fever (Sicilian) | ≧256† | ≧256 | ≧256 | ≧256 | ≧256 | <4 |
| Phlebotomus fever (Naples) | <4 | <4 | <4 | <4 | <4 | ≧256 |

* Negative results (reciprocal titer <4) from the CF test were obtained with the following hyperimmune sera: Western equine, Eastern equine, Venezuelan equine, Sindbis, Japanese encephalitis, Murray Valley, Russian spring-summer, West Nile, Dengue (type 1), and Qaranfil (AR1113).

† Numbers indicate reciprocal titer of antigen fixing complement in presence of 4 to 8 units of antiserum.

antigens prepared from the isolates and antisera against Naples type phlebotomus fever virus, Japanese encephalitis, Murray Valley encephalitis, Russian spring-summer encephalitis, West Nile, type 1 dengue, Qaranfil (AR 1113), Sindbis, Western equine, Eastern equine, or Venezuelan equine viruses.

It is of particular interest that the August and October isolates appear to be immunologically similar or identical, even though they differ markedly in their virulence for suckling mice. Minor differences among the isolates may perhaps be revealed in the more exhaustive antigenic comparisons now in progress.

*Virus-neutralizing capacity of sandfly suspensions.* By mid-August, over 40 pools of *P. papatasi* had been processed without evidence of an isolate, so the possibility was considered that minimal amounts of virus present in the suspensions were being neutralized by immune blood in engorged sandflies. Taylor[6] had previously demonstrated phlebotomus fever antibody (Sicilian type) in the sera of 5 to 43% of adult Egyptian villagers, and it was felt that antibody-containing blood in a portion of flies might be neutralizing virus in other flies when the two were ground together. To test this possibility supernates from 10% sandfly suspensions were tested in neutralization test fashion against varying dilutions of known Sicilian type phlebotomus fever virus (strain H 5202). In addition to suspensions of wild-caught female *P. papatasi*, suspensions of non-engorged laboratory-reared female *P. papatasi* and laboratory-reared male *P. papatasi* were tested for their neutralizing activity. The results of these tests, in which the supernates were allowed to stand in contact with virus for 45 minutes at room temperature, are presented in Table 4. It is apparent that engorged wild-caught *P. papatasi* contain substances that neutralize phlebotomus fever virus; that the neutralization is probably due to antibody in the blood of engorged flies is substantiated by the failure of non-engorged laboratory-reared flies to neutralize virus. Neutralization of 20, 2,000 and 4,000 mouse $LD_{50}$ of virus, respectively, by the three wild-caught pools tested, suggests that even though virus might have been present at high concentrations in these pools it would not have been detected by the isolation procedures employed. In fact, the suspensions tested for their neutralizing activity were portions of pools processed for isolation, all of which failed to yield virus.

TABLE 4

*Neutralizing capacity of sandfly suspensions for Sicilian type phlebotomus fever virus*

| Suspension | $LD_{50}$ of virus neutralized* |
|---|---|
| Wild-caught, engorged *P. papatasi* | |
| Pool 1 | 4,000 |
| Pool 2 | 2,000 |
| Pool 3 | 20 |
| Laboratory-reared, nonengorged female *P. papatasi* | ≦10 |
| Laboratory-reared male *P. papatasi* | 0 |

* After contact with suspension for 45 minutes at 21° to 25°C.

## DISCUSSION

The isolation of phlebotomus fever virus from wild-caught *P. papatasi* provides additional evidence of the role of this insect in the natural transmission of the disease. It does not necessarily prove, however, that the insect is a true biological vector. Since the majority of the sandflies processed for isolation in this study were partially or fully engorged, it is possible that they were merely acting as carriers of infected blood and that recovery of virus from them was no more than a demonstration of the prevalence of virus in the population on whom they had fed. Nevertheless, the work proves that *P. papatasi* under natural conditions does indeed carry the virus, regardless of the origin of that virus. The work of Sabin *et al.*[1] clearly demonstrated that once having fed on infected blood, *P. papatasi* may transmit the virus after a suitable extrinsic incubation period. Thus *P. papatasi* has literally been "caught with the goods", one of the cardinal requirements for proof of any insect as the natural vector of a disease.

## SUMMARY

A total of 24,179 female *Phlebotomus* flies collected in suburban Cairo, Egypt, were examined for phlebotomus fever virus. Virus was recovered from 4 of 59 sandfly pools tested; one pool of *Phlebotomus papatasi* and 3 pools of unindentified *Phlebotomus*, presumably consisting solely of *P. papatasi*, yielded virus. All four isolates were identified by complement-fixation

tests as the Sicilian type of virus. Wild-caught engorged *P. papatasi* flies were shown to contain substances which neutralize the Sicilian type of phlebotomus fever virus. Failure to demonstrate similar activity in laboratory-reared sandflies suggests that the active principle is antibody contained in ingested human blood.

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# STUDIES ON MALARIA IN CHIMPANZEES

## VIII. The Experimental Transmission and Pre-erythrocytic Phase of *Plasmodium malariae*, with a Note on the Host-Range of the Parasite*

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*Plasmodium malariae* has been transmitted experimentally by various species of *Anopheles* and its sporogony studied in the laboratory.[1-3] On the other hand only in one such case has *Anopheles gambiae* been infected with *P. malariae*[4] prior to work done at this Institute by Dr. R. C. Muirhead-Thomson and the author. Infection of *A. gambiae* here has been reported[5, 6] but more details of rates and intensity of infection can now be reported.

The primary aim of the infection of *A. gambiae* with *P. malariae* apart from determining the conditions under which infection and transmission are possible was to infect large numbers of mosquitoes and harvest their sporozoites for the demonstration of the pre-erythrocytic phase of *P. malariae*. Success in this aim is reported here.

*P. malariae* from both man and the chimpanzee were used in these experiments and transmission of strains of *P. malariae* from man to the chimpanzee was successful in several instances. It is now possible to give some indication of the host specificities of this parasite.

### MATERIAL AND METHODS

All lines of *P. malariae* were derived from natural infections of local inhabitants or of chimpanzees brought to the Institute from within Liberia.

The vertebrate hosts were man and *Pan troglodytes verus*, the local chimpanzee, and the invertebrate host was *A. gambiae* from a laboratory colony.

*A. gambiae* were fed on man by applying tubes of 50 mosquitoes to the legs, and on chimpanzees by applying tubes of 50 mosquitoes to the inner thigh and the shaven abdomen of ether-anaesthetized animals. The mosquitoes were kept at room temperature (approximately 27°C) or sometimes, at night only, slightly above room temperature. They were maintained on sugar solutions only before and after the blood meal.

Liver biopsies were made by open laparotomy. Liver pieces were fixed in Carnoy's fluid, Zenker's fixative and Bouin's fixative, sectioned and stained with Giemsa-colophonium, Giemsa-colophonium following hydrolysis with 1 N hydrochloric acid[7] and Feulgen-light green stain.

Infections in the vertebrate hosts were followed by thick blood films stained with Giemsa. Infection of chimpanzees was performed by the intravenous inoculation of infected blood or infected salivary glands of *A. gambiae* in 15 to 25% (v/v) inactivated human serum in Locke's fluid.

All chimpanzees used experimentally were treated with 300 mg chloroquine base daily for 3 days and 7.5 mg primaquine base daily for 14 days at least 2 months before use.

### RESULTS

*The infection of* A. gambiae *with* P. malariae *and transmission to the chimpanzee.* The results of various attempts to infect *A. gambiae* with gametocytes of *P. malariae* from man and chimpanzee and from lines in chimpanzees derived from man are shown in Table 1. As can be seen infections have been obtained when no microgametocytes were seen in man and no infections obtained when as many as 31 microgametocytes/cmm of blood were found in man.

No correlation between the blood picture and the resultant infection in *A. gambiae* has been found in these results. One subject having *P. malariae* microgametocytes in the blood off and on for 2 months was fed upon four times when microgametocytes were seen. On two occasions infections were obtained and on two occasions no infection resulted. It has not been possible to discover any definite correlation between infection of *A. gambiae* and the age of the gametocyte donor or the duration of infection in the

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TABLE 1

*Attempts to infect* A. gambiae *in the laboratory with* P. malariae

| Strain of gametocytes | Host of gametocytes | No. of micrograms per cmm | No. fed | No. guts dissected | No. positive | Avg no. oöcysts per infected gut | No. glands dissected | No. positive | Total percentage positive | Transmission *Successful* *Failed* |
|---|---|---|---|---|---|---|---|---|---|---|
| Human | Man | 7 | 60 | 26 | 6 | 4 | 16 | 8 | 33 | *S* |
| Human | Man | 13 | 76 | 10 | 1 | 3 | — | — | 10 | — |
| Human | Man | 10 | 50 | 18 | 8 | 2 | (6) | (2) | 45 | — |
| Human | Man | 2 | 40 | 14 | 0 | — | — | — | 0 | — |
| Human | Man | 0 | 50 | 35 | 3 | 2 | — | — | 9 | — |
| Human | Man | 9 | 50 | 30 | 5 | 2 | — | — | 17 | — |
| Human | Man | 7 | 50 | 17 | 0 | — | — | — | 0 | — |
| Human | Man | 18 | 50 | 6 | 4 | 2 | — | — | 67 | — |
| Human | Man | 19 | 50 | 21 | 1 | 1 | — | — | 5 | — |
| Human | Man | 15 | 475 | 62 | 14 | 1.5 | 10 | 4 | 25 | *S* |
| Human | Man | 7 | 50 | 21 | 4 | 2 | — | — | 18 | — |
| Human | Man | Scanty | 50 | 35 | 4 | 3.5 | — | — | 11 | — |
| Human | Man | 3 | 100 | 41 | 0 | — | — | — | 0 | — |
| Human | Man | 10 | 50 | 31 | 0 | — | — | — | 0 | — |
| Human | Man | 19 | 50 | 16 | 6 | 2 | 4 | 2 | 40 | — |
| Human | Man | 17 | 50 | 21 | 0 | — | — | — | 0 | — |
| Human | Man | 3 | 50 | 18 | 0 | — | — | — | 0 | — |
| Human | Man | 17 | 50 | 24 | 1 | 2 | — | — | 4 | — |
| Human | Man | 7 | 205 | 50 | 9 | 1.5 | — | — | 18 | — |
| Human | Man | 18 | 100 | 10 | 6 | 2 | — | — | 60 | *S* |
| Human | Man | 16 | 400 | 68 | 4 | 1 | — | — | 6 | — |
| Human | Man | 16 | 50 | 22 | 14 | 2 | — | — | 64 | — |
| Human | Man | 31 | 100 | 17 | 0 | — | — | — | 0 | — |
| Human | Man | 23 | 423 | 8 | 5 | 10 | 4 | 2 | 58 | *S* |
| Human | Man | 17 | 110 | 18 | 6 | 4 | — | — | 33 | *S* |
| Human | Chimp. | 1 | 305 | 71 | 0 | — | — | — | 0 | — |
| Human | Chimp. | 0 | 50 | 17 | 0 | — | — | — | 0 | — |
| Human | Chimp. | 2 | 100 | 43 | 0 | — | — | — | 0 | *F* |
| Human | Chimp. | 0 | 50 | 15 | 0 | — | — | — | 0 | — |
| Human | Chimp. | 9 | 62 | 13 | 0 | — | — | — | 0 | — |
| Human | Chimp. | 22 | 900 | 33 | 0 | — | — | — | 0 | — |
| Human | Chimp. | 13 | 592 | 27 | 1 | 1 | — | — | 4 | — |
| Human | Chimp. | 12 | 325 | 31 | 0 | — | — | — | 0 | — |
| Human | Chimp. | 21 | 450 | 100 | 6 | 1.5 | — | — | 6 | — |
| Chimp. | Chimp. | 2 | 100 | 27 | 0 | — | — | — | 0 | — |
| Chimp. | Chimp. | 47 | 750 | 83 | 0 | — | — | — | 0 | — |
| Chimp. | Chimp. | 5 | 100 | 23 | 0 | — | — | — | 0 | — |
| Chimp. | Chimp. | 19 | 375 | 43 | 0 | — | — | — | 0 | — |

donor. In all cases of human strains in chimpanzees the animals were splenectomized as only then do sufficiently heavy infections appear that could produce any number of gametocytes. The normal course of such infections is the production of no microgametocytes whatsoever. The cases listed are from 3 chimpanzees only, four cases from each of 2 chimpanzees and one from another. Altogether 10 chimpanzees have been infected with human strains and in seven of these post-splenectomy heavy infections of between 25,000 and 50,000 parasites/cmm of blood no microgametocytes were seen.

In the case of *P. malariae* of chimpanzee origin, in one case at least, all circumstances led to the belief that a reasonable infection could be obtained in a susceptible mosquito. The complete failure to infect *A. gambiae* under these circumstances may be significant when it is considered that *A. gambiae* can be infected with

a human strain of *P. malariae* whether the donor is man or chimpanzee.

In general the infection of intact (*i.e.*, unsplenectomized) chimpanzees with sporozoites or trophozoites of *P. malariae* derived from man never gave rise to heavy infections (not higher than 100 parasites/cmm).

In all, 78 intact chimpanzees have been examined for natural infections and five have shown *P. malariae*; in no case was the infection of an order higher than 100 parasites/cmm pre-splenectomy, nor were gametocytes ever seen pre-splenectomy. One further infection with *P. malariae* was revealed by splenectomy making a total of six infections with *P. malariae* seen among 78 animals.

On two occasions sufficient mosquitoes with medium infections were available for attempts to demonstrate the pre-erythrocytic cycle of *P. malariae*. On one occasion 110 salivary glands of *A. gambiae* 50% infected with sporozoites of *P. malariae* were inoculated into an intact chimpanzee (No. 55) and another 110 glands from the same batch inoculated into a splenectomized chimpanzee (No. 61). On another occasion 72 salivary glands of *A. gambiae* 33% infected with sporozoites of *P. malariae* were inoculated into a splenectomized chimpanzee (No. 59). At the same time 110 uninfected salivary glands were inoculated into chimpanzee No. 33.

Liver biopsies or liver pieces at autopsy were taken from No. 55, 9 and 11 days after infection; from No. 61, 8 and 10 days after infection; from No. 59, 12 and 12.5 days after infection; and from No. 33 (control), 10 days after inoculation.

All of the liver pieces from infected chimpanzees showed pre-erythrocytic forms of *P. malariae* which are described below and in Table 2.

*The 8 day-old pre-erythrocytic schizonts* (Fig. 1). The parasite lay in a parenchyma cell usually in a vacuole which may be the result of Carnoy fixation as it was not always evident (Fig. 1). The host cell was enlarged and its cytoplasm was often pushed aside and piled up in a crescent shape around the parasite. At the center of this crescent of host cell cytoplasm lay the host cell nucleus. The cytoplasm was not always evident and occasionally the host cell cytoplasm was tenuous but in contra-distinction to pre-erythrocytic schizonts of most other species the impression was left that the host cell cytoplasm was not being used as food by the parasite to any great extent.

The host cell nucleus (or nuclei) was enlarged and pushed to one side (Fig. 1); in 15 of the 27 parasitized cells seen two or more enlarged nuclei were found in the host cell. It was difficult to determine in sections cut at 4 $\mu$ whether this was an unusually high proportion of double nucleated cells but the general impression was that in the chimpanzee at least half of the parenchyma cells of the liver were multinucleate so that

TABLE 2

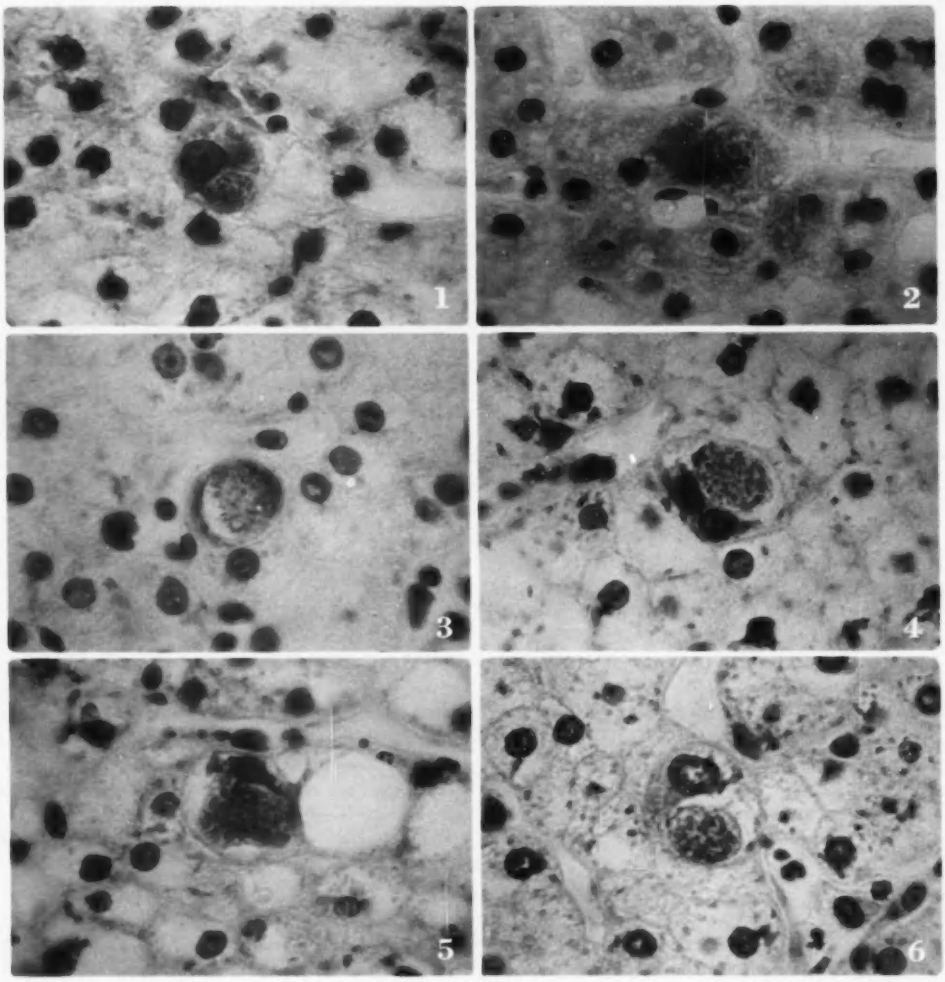
*Pre-erythrocytic schizonts of* P. malariae

| Chimp no. | Age of schizont | No. exam. | Avg diam. in $\mu$ | Avg no. of nuclei | Avg diam. host cell in $\mu$ | Avg diam. liver cell* in $\mu$ | Avg diam. host cell nucleus in $\mu$ | Avg diam. liver cell nucleus* in $\mu$ |
|---|---|---|---|---|---|---|---|---|
| 61 | 8 | 27 | 12.5 (9.5–17) | 25 (7–49) | 28.3 (23–36) | 20.7 (13–28) | 11.0 (8.5–13) | 8.3 (4.5–9.5) |
| 55 | 9 | 15 | 12.7 (8.5–21) | 27 (10–83) | 25.4 (21–30) | 19.7 (13–26.5) | 11.1 (8.5–13) | 7.8 (5–10) |
| 61 | 10 | 50 | 22.1 (15–29) | 100.1 (33–201) | 33.5 (28–38) | 20.7 (13–28) | 11.7 (8.5–17) | 8.3 (4.5–9.5) |
| 55 | 11† | 29 | 11.7 (9.5–14) | 14.8 (6–30) | 29.9 (22.5–36) | 19.7 (13–26.5) | 11.5 (7.5–15) | 7.8 (5–10) |
| 59 | 12 | 19 | 41.0 (34–47) | 712 (435–1151) | 44.6 (36–49) | 20.1 (13–29) | 12.9 (11–19) | 7.9 (5–10) |
| 59 | 12½ | 11 | 48.0 (43–51) | 1537 (1294–1739) | 52.9 (46–57) | 20.1 (13–29) | 12.7 (11–14) | 7.9 (5–10) |

Ranges in parenthesis.

* Average of 500.

† These figures do not include one schizont described in the text.




All figures are untouched Kodachrome color photomicrographs of sections of pre-erythrocytic forms of *Plasmodium malariae* using the Zeiss Standard microscope and camera attachment Basic Body No. 2, with 8 × eyepiece and 100 × objective. All parasites are stained with Giemsa stain and colophonium differentiation.

FIG. 1. 8 day-old form. Note the enlarged host cell nucleus.

FIG. 2. 9 day-old form. Note the cytoplasm of the host cell pushed to one side and the distinct parasite envelope.

FIG. 3. 10 day-old form. The focus of the photomicrograph is on the few nuclei showing the 3 and 4 point structure.

FIG. 4. 10 day-old form. Note the enlarged host cell nuclei and the 3 point nuclei of the parasite

FIG. 5. 10 day-old form. Note the slight peripheral vacuolation

FIG. 6. 10 day-old form. Note the enlarged host cell nucleus and the pushed aside host cell cytoplasm, also the distinct parasite envelope.

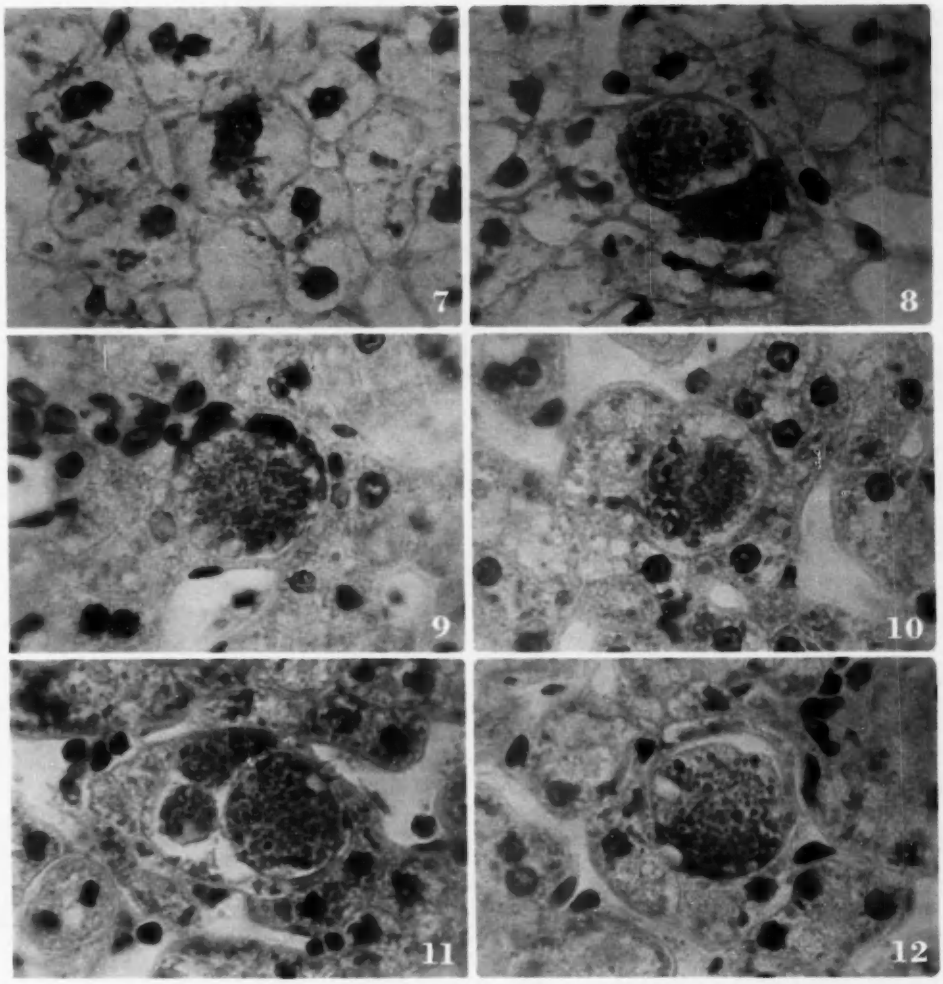



FIG. 7. 11 day-old form. Note the small size of the parasite but the considerable enlargement of the host cell nucleus.

FIG. 8. 11 day-old form. This is the "normal" schizont as described in the text. Host cell cytoplasm is pushed aside and the host cell nucleus much enlarged.

FIG. 9. 12 day-old form. Note the extensive peripheral vacuolation.

FIG. 10. 12 day-old form. Note the slight peripheral vacuolation and the enlarged host cell nuclei.

FIG. 11. 12.5 day-old form. Note the extreme lobulation showing as three islands which are transverse sections through 3 lobes.

FIG. 12. 12.5 day-old form. Note the large peripheral vacuoles.

preferential invasion of multinucleate cells was not occurring. Very occasionally large parenchyma cell nuclei were encountered but none was as large as the largest host cell nuclei of parasitized cells and thus preferential invasion of cells with large nuclei probably did not occur.

There was an impression that the host cell wall is thickened but careful examination did not establish this as a fact as some cytoplasm belonging to the cell and the surrounding cells was always compressed against the cell wall.

The parasite itself usually had a distinct envelope with no clefts. The cytoplasm could be tenuous, in strands, but more often was homogenous and drawn slightly back from the envelope, sometimes leaving nuclei in the clear space at the periphery. Occasionally the periphery showed a series of tiny vacuoles and on one occasion a vacuole of 2.5 $\mu$ in diameter was found at the periphery. The nuclei varied in diameter between 0.5 and 1.5 $\mu$ but usually were seen as either a small dot of about 0.5 $\mu$ or as a round compact deeply staining mass of chromatin about 1 $\mu$ in diameter. In about one quarter of the parasites some nuclei could be seen to be made up of three or four dots. These dots were joined by lightly staining strands. The dots were deeply staining and in several cases, presumably due to damage, were seen strung out in a line.

*The 9 day-old pre-erythrocytic schizonts* (Fig. 2). The observations on the host cell made for the 8 day-old schizont hold true in this case (Fig. 2). The parasite possessed a very distinct and complete envelope. The scatter in size of the parasite was greater than that of the 8 day-old schizonts, one parasite being smaller and one parasite larger than any of the 8 day-old forms. Other than this these parasites resembled exactly the 8 day-old schizonts. (Fig. 2).

However a distinct distribution of the schizonts was noted. The 8 day-old forms were scattered throughout the tissue examined. In the case of the 9 day-old forms it was noticed that there was a distinct grouping. Three or four schizonts would be found in 15 to 20 sections of 4 $\mu$ thickness and then none would be found for 30 to 50 sections. These three or four schizonts were within 0.5 mm of each other in the section surfaces. All except two of the schizonts seen were in four distinct "nests" of this type. Distribution in relation to the whole liver could not be determined as the tissue examined was obtained by biopsy of the edge of the right lobe.

*The 10 day-old pre-erythrocytic schizonts* (Figs. 3–6). The parasite lay in a parenchyma cell usually in a vacuole and its presence had pushed the enlarged nucleus or nuclei well to one side of the enlarged host cell (Figs. 4, 6). Once again much of the cytoplasm of the host cell was piled up into a crescent around the parasite (Fig. 6). Two parasites were found in cells surrounded by a small area of other parenchyma cells containing large empty vacuoles. One parasite was in a cell with one such vacuole somewhat distorted by the presence of the parasite. While the preparation of the sections did not allow fat staining procedures, there can be little doubt that these vacuoles had contained fat and thus liver cells containing abnormal amounts of fat would appear to be prone to invasion by the parasite.

The parasite possessed a distinct envelope and in about 50% of the parasites the cytoplasm was homogenous, reasonably dense and pulled slightly back from the envelope. In about 40% the cytoplasm was similar but not contracted away from the envelope. The remaining 10% were a few parasites which possessed a cytoplasm irregularly vacuolated with a few tiny vacuoles (Fig. 5) and two with a bubbly periphery consisting of small vacuoles. Most of the nuclei were round with about a quarter of the parasites showing a few three- or four-point type nuclei (Figs. 3, 4). The nuclei were of about the same size in all schizonts irrespective of age and size.

*The 11 day-old pre-erythrocytic schizonts* (Figs. 7, 8). The host cells are similar to those of the 8 and 9 day-old forms (Fig. 7). If the not unreasonable assumption is made that the 8 and 10 day-old forms from chimpanzee No. 61 display normal development then it can be seen from Table 2 that the 11 day-old forms seen are abnormal in size and rate of development. In fact the 11 day-old forms resembled the 8 day-old forms in all particulars except for one schizont which is not included in the averages for 11 day-old forms to be found in Table 2. This schizont (Fig. 8) was excluded as it is considered to show normal development and will be described here.

The parasite (Fig. 8) had a distinct envelope with an homogenous centrally situated cytoplasm except for three small cytoplasmic aggregations. The periphery was vacuolated at some places. The nuclei were mostly round with a few three- or four-point type nuclei. The parasite lay in a vacuole in the enlarged host cell whose cytoplasm and enlarged nucleus were pushed to one side.

The schizont was spherical, measured 36 $\mu$ in diameter and contained 221 nuclei. The host cell diameter was 41.5 $\mu$ and the host cell nucleus diameter was 18 $\mu$.

The 11 day-old forms were found in liver pieces taken from various parts of the liver at autopsy. Some evidence was gained of a distribution phenomenon. In a piece taken from the center of a lobe only one schizont was found. In a piece taken from the surface of a lobe four schizonts were found and all were proximal to the surface side of the section, *i.e.*, that side showing a connective tissue lining. The remainder of the schizonts were found in two pieces of tissue taken from the edge of a liver lobe, the triangular sections of which showed connective tissue lining two sides. About one-third of these schizonts were found in an area at the apex of the triangle where the connective tissue lined edges met.

*The 12 day-old pre-erythrocytic schizonts* (Figs. 9, 10). The host parenchyma cell was much enlarged and, even at this stage in several, most of the host cell cytoplasm was unused, pushed aside and piled up to one side with the nucleus. The host cell nucleus was enlarged but not much more so than that of the 10 day-old forms (Fig. 10).

The parasite displayed a distinct envelope consisting of a series of arcs of the vacuoles lining the periphery (Fig. 9). The cytoplasm was dense in the central portions of the parasite but the periphery of the parasite consisted of vacuoles varying in diameter from 3 to 6 $\mu$ (Fig. 10). These vacuoles had very distinct outlines and contained lightly staining eosinophilic material. The cytoplasm contained a number of small basophilic aggregations (Fig. 9). The nuclei resembled those of the earlier schizonts. The parasite was sometimes slightly lobulated as two very blunt short lobes.

*The 12.5 day-old pre-erythrocytic schizonts* (Figs. 11, 12). The host cell was altered in the same manner as that of the 12 day-old forms. The parasite showed a distinct envelope and otherwise resembled the 12 day-old form qualitatively except in two characteristics. The parasite could be markedly lobulated (Fig. 11). One schizont was seen to have four distinct and extensive lobes and in fact the common mass was small compared with the lobular masses. Most schizonts showed some lobulation. Also the peripheral vacuolation was most marked (Fig. 12). Large peripheral vacuoles of 5 to 8 $\mu$ were the rule and these contained quite dense eosinophilic material.

In general several further observations should be made about all of the schizonts. In no instances at any age did any but random distribution of nuclei occur and no "pseudo-cytomeres" or evidence of septa formation, vacuole anastomosis and plasmotomy was seen as in the schizonts of *P. inui*. Invasion of multinucleated cells was common: 63% of all schizonts were found in multinucleated cells. As far as could be determined, about 60% of normal liver cells were multinucleate.

Giemsa stain following hydrolysis did not allow any clearer idea of the nature of the nuclei. There is no doubt that some consisted of very small dots of chromatin. Rarely these could be seen to be four but more often they appeared as three dots in one plane of vision and sometimes there was the suspicion of a fourth dot in another plane. Schizonts 10, 12, and 12.5 days old were stained with Feulgen's stain. Some nuclei in all schizonts at these ages were Feulgen positive. The intensity of the stain was never great and was much the same as that obtained in the schizonts of *P. vivax* and *P. cynomolgi*,[8, 9, 10] but was not as intense as that shown by *P. inui* pre-erythrocytic schizonts.[8] Intensity ranged from barely visible in 12 day-old schizonts to distinct but light in 12.5 day schizonts with the 10 day forms intermediate.

At no time were any mature schizonts seen or any areas of local reaction containing merozoites or pieces of schizonts. One area of local reaction similar to those seen following the bursting of a pre-erythrocytic schizont was seen but no merozoites could be found in this area. In the surviving (splenectomized) chimpanzee which received 110 glands the blood became positive 16 days after inoculation. The blood of the splenectomized chimpanzee which received 72 glands became positive 18 days after inoculation. It is not possible to say what the duration of the pre-erythrocytic generation is.

The entire piece of liver from chimpanzee No. 33, which received 110 uninfected glands intravenously, was sectioned and examined. No schizonts were found. One hundred sections of liver from chimpanzee No. 33 and an equivalent area of sectioned liver from chimpanzee No. 61 taken 10 days after infection were stained and searched by a technician who had been deliberately kept in ignorance of the details of the experiments. He reported 14 schizonts in the material from chimpanzee No. 61 and no schizonts in the material from chimpanzee No. 33.

## DISCUSSION

Shute and Maryon[2] stated "it is clear that less is known about the transmission of *Plasmodium malariae*, either in nature or in the laboratory, than about the other species of human malaria parasites." Despite the reasonably extensive study on the transmission reported here this statement still stands. The present study allows only one conclusion: if a relatively large number of microgametocytes of *P. malariae* are present in the blood of man on which *A. gambiae* is fed, the mosquito probably will become infected. Two impressions were gained but are unsupported by any conclusive data; first, infection in *A. gambiae* is more likely to occur if the microgametocytes are found in an adult and second, infection is more likely to result from the gametocytes which appear relatively early in the course of the infection.

Shute and Maryon[2] also stated "in our experience it is not always possible to distinguish a female gametocyte in a stained film from a fully grown asexual parasite." I would like to put this more strongly and state that it is never possible to distinguish a macrogametocyte as such using Romanowski stains. It may be possible in a closely studied, highly synchronous infection to state that at a certain time all asexual parasites will be in the ring stage and thus identify a large parasite as a macrogametocyte. In the normal naturally acquired infection and in induced infections in splenectomized chimpanzees, however, such synchronism does not occur. In these cases large parasites filling the whole cell with a compact nucleus and scattered pigment are frequently seen when microgametocytes are absent and remain absent, and these parasites themselves are frequently in much lesser numbers the following day.

On the other hand the microgametocyte is recognizable in thin films; in Liberia it is even more definite and recognizable in thick films. The thick film dries slowly in the humid climate of Liberia and the parasite partially disintegrates to form a pink mass containing pigment and a large round red nucleolus which is highly distinctive.

The relationships between man, chimpanzee, *A. gambiae* and their malaria parasites are now partially clear. Dealing with the genus *Laverania* neither man nor *A. gambiae* is susceptible to *L. reichenowi* of chimpanzees.[11, 12] The chimpanzee liver is susceptible to *L. falciparum* of man and *L. falciparum* invades erythrocytes of an intact chimpanzee but is rapidly eliminated from the blood. The splenectomized chimpanzee allows the development of *L. falciparum* in the blood.[12] *A. gambiae* is susceptible to *L. falciparum*.

Dealing with the genus *Plasmodium*, man and *A. gambiae* are partially susceptible to *P. vivax schwetzi* of chimpanzees; man can be infected by blood inoculation and will carry the infection for rather less time than is the case with *P. vivax vivax*;[14] *A. gambiae* can be infected in the gut but eliminates the sporozoites once these are released.[15] The chimpanzee liver is susceptible to *P. vivax vivax* and the blood can be infected at sub-microscopic levels which may persist for at least 3 months. The splenectomized chimpanzee allows the development of *P. vivax vivax* in the blood.[9] *A. gambiae* is susceptible to *P. vivax vivax*.[9]

The chimpanzee liver is susceptible to *P. ovale*, but *P. ovale* is eliminated in the blood in the same manner as is *L. falciparum*.[16] The splenectomized chimpanzee allows the development of *P. ovale* in the blood.[16] *A. gambiae* is susceptible to *P. ovale*.[16]

*A. gambiae* is not susceptible to *P. malariae* of chimpanzee origin but man is.[11] The chimpanzee liver is susceptible (though perhaps not completely) to *P. malariae* of man. Chimpanzee blood is susceptible to *P. malariae* of man though in my experience it is less susceptible than man. Until the susceptibilities of the vector of the quartan malaria parasite of chimpanzees are known it seems as well to allow the name *P. malariae* to apply to the quartan malaria parasites of both man and chimpanzees.

Thus the relationship between the vertebrates is a gradation of susceptibility to cross-infection ranging from insusceptibility with the *Laverania* to susceptibility with *P. malariae*. *A. gambiae* is insusceptible to the complete sporogony of all chimpanzee malaria parasites. Two major gaps in the knowledge of this complex remain. Nothing is known of the susceptibility of splenectomized man or the liver of man to chimpanzee malaria parasites. This may remain unknown for some time. Nothing is known of the vector of chimpanzee malaria parasites.

Two points of some interest should be discussed in the light of these relationships: first, the question of a zoonosis of *P. malariae* infection.

While a zoonosis, and therefore a threat to man not covered by present methods of malaria eradication, is possible due to the reservoir of *P. malariae* infections in chimpanzees and man's susceptibility to these parasites, it would not appear to be probable. The present indications are that the vectors of human malaria which live in close relation to man and his dwellings are and will prove to be insusceptible to *P. malariae* of chimpanzee origin. The probability is that the exclusive vectors of chimpanzee malaria are forest anopheles not in contact with man and only a specialized activity of man, like that involved in the epidemiology of muco-cutaneous leishmaniasis which takes him to the forest and probably to the upper levels of the forest, is likely to involve him in a zoonosis of *P. malariae*. While this possibility cannot be ruled out it should not be considered as a serious one at this time. As chimpanzee baited traps on bush tracks between villages in Liberia have caught *A. gambiae* and *Aedes aegypti* only, it can be said that at present *P. malariae* of man represents a greater threat to the chimpanzee than *P. malariae* of chimpanzees does to man.

Secondly, it was hoped that these relationships might throw some light on the origin and phylogeny of the malaria parasites of man. Apart from the probability that the parasites infected some common ancestor of man and chimpanzee and have been harbored by man and the chimpanzee during their existence as such, little can be said.

It is assumed and often categorically stated that *L. falciparum* is of recent origin and *P. malariae* is ancient. These statements have been vaguely based on virulence and imprecise notions about senescence and the waning ability to multiply at a high level. More recently *Laverania* as a genus has been claimed to be of recent origin because of its further step from tissue habitat to blood habitat.[1]

However the relationships described above show that *Laverania* has completely separated into species in the two environments of man and chimpanzee, while *P. malariae* has not separated into even varieties in these environments though its separation in its invertebrate hosts might be considered to confer varietal status. Thus if time were the prime factor in the emergence of speciation in specific environments then it could be argued that *Laverania* is ancient while *P. malariae* is of recent origin. However as *Laverania* populations are always much larger, time is not of prime importance and it is necessary to assume that the common ancestor of man and the chimpanzee harbored both *Laverania* and *P. malariae*. Any further look backwards is blocked by the presence of *P. malariae* in man and higher apes in Africa but the absence of a quartan malaria parasite in the lower apes of Africa, and there speculation must stop.

*P. malariae* has proved itself quite distinct in its pre-erythrocytic phase. The schizonts described not only differ markedly from those of other species of human malaria parasites but also from those of *P. inui*, the quartan malaria parasite of Asian macaques.[17] It becomes obvious that prediction of morphology in the tissue phase is a most doubtful procedure now that *P. gonderi*, the tertian parasite of African monkeys, has been shown to differ considerably in the morphology of its pre-erythrocytic schizonts from *P. cynomolgi*, the tertian parasite of Asian monkeys, and from *P. vivax*.[18] This added evidence of considerable variation between *P. malariae* and *P. inui* only increases the belief that prediction is not useful.

*P. malariae* is distinct from *P. vivax* and *Laverania falciparum* in the tissue phase in that it causes enlargement of the host cell nucleus and the older forms show considerable vacuolation of their peripheral cytoplasm. It produces fewer merozoites and its growth rate is considerably slower. It is also distinct from *P. ovale* in the tissue phase in that it and its nuclei are smaller, it produces smaller and fewer merozoites and its growth rate is somethat slower. It is surprisingly distinct from *P. inui* in that it is twice as large in its later stages, it shows no patterning of nuclei or "pseudo-cytomeres," it displays the distinctive vacuolation at the periphery of the older forms, and it causes enlargement of the host cell nucleus.

The large peripheral vacuoles containing eosinophilic material seen in the 12.5 day-old forms are reminiscent of the large chromatin masses seen in the second generation tissue schizonts of *P. ovale* while the lesser peripheral vacuolation in the 12 day-old forms is similar to that seen in 7 day-old pre-erythrocytic schizonts of *P. ovale*.[16]

There was considerable variation in growth between the schizonts demonstrated in the two splenectomized chimpanzees on the one hand and

those produced in the intact chimpanzee on the other. In the splenectomized animals schizogony proceeded in the expected manner giving a four fold increase in diameter between schizonts examined after 8 days and those examined after 12.5 days of growth whereas in the intact chimpanzee no increase in diameter occurred between schizonts examined after 9 days and those examined after 11 days of growth. Further, the predicted (and in one case actual) diameter of 11 day-old forms was 35 $\mu$ whereas the actual diameter of all forms except one from the intact chimpanzee averaged 11.7 $\mu$. The 9 day-old forms taken from the intact chimpanzee showed no increase in size over the 8 day-old forms taken from a splenectomized chimpanzee.

Further the 9 and 11 day-old forms from the intact chimpanzee showed a distinct tendency to appear close to the outer surfaces of the liver or in nests, whereas all forms from the splenectomized chimpanzees showed a random distribution in the liver. As only one intact chimpanzee was involved it is impossible to draw any conclusions from this information.

## SUMMARY

*Anopheles gambiae* was fed on human subjects and on chimpanzees harboring *Plasmodium malariae* on 38 occasions. In many cases the mosquitoes became infected and on 5 occasions sporozoites of *P. malariae* of human origin were transmitted successfully to chimpanzees. No correlation between observed gametocytemia and infection in the mosquito was found.

All attempts to infect *A. gambiae* with *P. malariae* of chimpanzee origin failed, but *A. gambiae* could be infected by feeding on chimpanzees harboring *P. malariae* of human origin.

Pre-erythrocytic schizonts of *P. malariae*, 8, 9, 10, 11, 12 and 12.5 days old are described from the liver of chimpanzees. They lie in parenchyma cells, enlarge the host cell nucleus, show a peripheral vacuolation and in general are distinct from similar forms of other species of malaria parasites.

The pre-erythrocytic schizonts of *P. malariae* found in one chimpanzee were apparently abnormal, being much smaller than normal, and they displayed a distinct distribution in the liver, being found in "nests" and close to the outside surfaces of the liver.

The appearance of the gametocytes of *P. malariae* and their infectivity to *A. gambiae* are discussed. It is concluded that, while the microgametocyte of *P. malariae* is distinctive, the macrogametocyte cannot be distinguished. No conclusions about the infectivity of these gametocytes were possible.

The interrelationships between man, chimpanzee, *A. gambiae* and their malaria parasites are described and discussed in relation to a possible zoonosis involving *P. malariae* and in relation to the phylogeny of the human malaria parasites. It is concluded that while a *P. malariae* zoonosis is possible it does not constitute an important threat at present.

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## BOOKS RECEIVED

*Carter's Microbiology and Pathology*, by Alice Lorraine Smith, A.B., M.D. 7th edition, 742 pages, ill. St. Louis, The C. V. Mosby Co., 1960. $7.50.

*Anatomy. A Regional Study of Human Structure*, by Ernest Gardner, M.D., Donald J. Gray, Ph.D., and Ronan O'Rahilly, M.Sc., M.D. 1st edition, 999 + xvi pages, ill. Philadelphia and London, W. B. Saunders Co., 1960. $15.00.

*Seuchen im Menschen* (in German), by Prof. Dr. Erich Martini. 1st edition, 399 + iii pages. Stuttgart, Ferdinand Enke Verlag, 1959. DM 35 (paper DM 32).

*A Handbook of Diseases of the Skin*, by Henry O. Mackey, F.R.C.S.I., L.R.C.P.I., D.P.H. (Univ. Dub.), L.M., F.R.I.A.M. 6th edition, 263 + x pages, ill. Dublin, C. J. Fallon L'td., 1959. 8/6.

*Doctor in Bolivia*, by H. Eric Mautner, M.D. 331 + viii pages. Philadelphia, Chilton Co., 1960. $5.95.

*Manual of Tropical Medicine*, by George W. Hunter, III, Ph.D., William W. Frye, Ph.D., M.D., Sc.D. (Hon.) and J. Clyde Swartzwelder, Ph.D. 3rd edition, 892 pages, ill. Philadelphia, W. B. Saunders Co., 1960. $15.00.

*Medizinische Mikrobiologie, Immunologie und Epidemiologie*, Teil I., by Prof. Dr. Med. Habil Georg Wildführ. 1st edition, 619 + xv pages, ill. (color). Leipzig, VEB Georg Thieme, 1959. DM 86.60.

## BOOK REVIEWS

**Immunization Information for International Travel.** (Public Health Service Publication No. 384.) 77 pages, illustrated (maps). Washington, D. C., Government Printing Office, 1959. $0.30.

This booklet is a useful, almost necessary, reference for persons intending to travel outside the Country. After a general statement of the policy of the United States and other nations concerning immunization and of inoculation schedules, it presents an authoritative country-by-country list of requirements and recommendations for entry and through passage. It also lists the designated yellow fever vaccination centers in the United States and gives a summary of U. S. Public Health Service regulations governing the importation of pet animals. This latest revision brings all of these statements up to date, and is highly recommended reading.

HENRY M. GELFAND

**Microbial Variation.** V. D. TIMAKOV, editor. (Translated from the Russian by G. H. BEALE) 1st edition, 202 pages, illustrated. New York, N. Y., Pergamon Press, 1959. $6.50.

In 1948, at the Lenin All-Union Academy of Agricultural Sciences conference held in Moscow, T. D. Lysenko announced that the Central Committee of the Communist Party had examined his theory and approved it. There followed a series of confessions of ideological error by many of the attending scientists (Lysenko, T. D., 1949. The Situation in Biological Science. *Proc. Lenin Acad. Agric. Sci. U.S.S.R.*, July 31–Aug. 7, 1948, Foreign Languages Publishing House, Moscow). Another aftermath was the adoption of Lysenko's theories by the Gamaleia Institute of Epidemiology & Microbiology whose workers set out "to find *whether* directed variations can in principle be made to occur, *in order* to obtain avirulent strains having immunizing ability." Consideration of this intention should give some idea of the objectivity involved in these investigations but, nonetheless, directed variations were sure enough directed, unless, of course, the facts reported are simply red herrings drawn across the path of Western Science.

To this reviewer the facts are, on the contrary, plausible. Repeated subculture in media of special character, such as that containing disintegrated or heat-killed bacteria of another species, is bound eventually to yield populations of organisms unusual enough to confound the bacterial taxonomist. One does not have to be a Soviet scientist to accomplish this. This is especially so when opportunities are presented for transformation by nucleic acids, for transduction by bacteriophage, and for whatever as-yet-undiscovered mechanisms of infectious heredity may exist in bacterial systems. Nothing claimed in this book is startling but the pain taken to secure and characterize new populations is impressive. Perhaps comparable efforts should more often be made in Western industrial and medical laboratories.

Here they could be made much more intelligently in the framework of a better understanding of the facts and principles of bacterial heredity and evolution than the Russians appear to have.

Timakov concludes: "The essential basis of a micro-organism, its nature or heredity, which has developed during the historical process of evolution and been determined by the conditions under which it has existed, may vary when it is subjected to the action of qualitatively different conditions of existence and is compelled to assimilate unfavorable food substances." According to Michurin, "variation is caused by the development of an organism under environmental conditions which in some way or another do not correspond with the natural requirements of the given organic form." Such cloudy concepts do not match in adroitness those of the English Lamarckian, Sir Cyril Hinshelwood. The Russians feel the need to contrast their "progressive materialistic" position with the "idealistic" one of Western scientists who are reported to consider "that micro-organisms do not change their hereditary basis or nature." Apparently a Soviet scientist must at least seem to accept such materialistic concepts as T. D. Lysenko's definition of a species ("a particular qualitatively-defined state of the living forms of matter"), his notion of the unity of organism and environment and Michurin's methods of treating the "nature" of an organism, whatever that may be. Michurin, like Luther Burbank and the 19 authors of this volume, may have bent Nature to their wills but *her* nature has escaped them. Although the words "selection," "Avery," and "bacteriophage" appear in this book there is no indication that they are understood. Certainly the experiments reported are not designed to reveal variation as an event that occurs within a cell, as distinguished from variation resulting from population dynamics. Even when nucleic acids or bacteriophages are used to induce changes there is no discussion of the brilliant work done elsewhere in the world on transformation, transduction or lysogenic conversion which are among the incisive scientific glories of this "space age." Further, although controls in experiments are not necessary to achieve results, they help in their interpretation; controls are infrequently referred to in this book. Here we might recall the terror mentioned at the beginning of this review and ponder the adage "There but for the grace of God, go I."

As would be expected of Dr. Beale, the translation reads well and seems made in fairness. It might have been helpful for the publishers to indicate the year the work appeared in Russian; it was 1956.

FRANCIS J. RYAN

**Live Poliovirus Vaccines** (Scientific Publication No. 44), by various authors (A Symposium). 713 pages, illustrated. Washington, D.C., Pan American Sanitary Bureau, 1959. $5.00

A five-day conference dealing with the status of live poliovirus vaccines was held under the auspices of the World Health Organization and the Pan American Sanitary Bureau in June, 1959. Although the conference was organized because of pressures on the sponsoring agencies to provide advice on the use of live polio vaccines, the objective of the meeting was not to make recommendations but to marshal pertinent data and to elicit differences of opinion in order to define areas requiring further investigation. The participation of the proponents of the three different sets of live virus strains, of individuals responsible for field trials that already have involved administration of oral vaccine to some millions of people, and of a distinguished group of virologists, epidemiologists, and public health administrators provides an interesting 692-page conference record that summarizes the knowledge of live poliovirus vaccines as of mid-year 1959. The record also conveys the atmosphere of mixed scientific detachment and emotional attachment that pervaded the discussions.

The proceedings were organized under five topic headings. Under "General Considerations", G. W. A. Dick played the role of a "devil's advocate" in stressing the problems inherent in the proper evaluation of live poliovirus vaccines. Seven papers were presented under the topic heading "Criteria of attentuation, development, selection, and testing of poliovirus strains for use in field trials." Topic III, "Properties and behavior of orally administered attenuated strains" and Topic IV, "The problem of interference in live poliovirus immunization" are each represented by six formal contributions. The fifth topic is concerned with actual field trials and laboratory studies thereon and is composed of 19 papers.

Interspersed between the various groups of papers are some 78 pages of discussion. Brief comment is made with reference to the problems of licensing live poliovirus vaccines in the United States. Although the field is changing rapidly and will be the subject of another conference in 1960, this report should be required reading for all whose official or professional obligations relate in any capacity to the matter of live poliovirus vaccine field trials. Further, it will be of interest to most virologists as a summary of one important aspect of current work on the polioviruses.

THOMAS H. WELLER

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